# EFFECTS OF CONVENTIONAL AND MICROWAVE TREATMENTS ON INHIBITORY ACTIVITY AND STRUCTURAL CONFORMATION OF TRYPSIN INHIBITOR PROTEIN

BY

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## ABSTRACT

Food processing and preservation techniques provide high quality nutritive food, reduce the risk of contracting food-borne diseases and increase the shelf life of food products. Processing techniques further increase the digestibility of food, resulting in greater bioavailability of the nutrients and also in the elimination of undesirable factors. Though extensive studies have been performed using various commercial processing methods such as sterilization, pasteurization, autoclaving, extrusion, homogenization, blanching, batch boiling, steam injection, ultra-high temperature (UHT), high temperature and pressure combination, ohmic heating and high pressure processing (HPP), for producing quality food, limited knowledge is available in terms of the effects of processing at atomic and molecular level. Thus, the studies to understand the changes at molecular level could help industrialists to design processing methods with increased nutritive and sensory appeal of food products.

In this study, the effects of conventional thermal and microwave treatments on raw soymilk samples for the inactivation of soybean trypsin inhibitors were evaluated. *In- vitro* protein digestibility (IVPD) studies were also conducted for understanding the effects of aforementioned processing techniques on soymilk proteins. The levels of microwave processing ($70^0$C, $85^0$C and $100^0$C for 2, 5, 8 min) and conventional treatment ($70^0$C, $85^0$C and $100^0$C for 10, 20, 30 min) were optimized using Response Surface Methodology (RSM).

Molecular dynamics (MD) simulation modelling techniques have been applied to explore and visualize the behavior of proteins, at ambient conditions and under the influence of external electric field stress, temperature and time. Soybean trypsin inhibitor (STI) protein was selected for MD simulations. By performing these simulations under the electric field strength of 0.5 V/nm, a valuable insight into the effects of electric field on protein conformation was noted and evaluated

using through Root Mean Square Deviation (RMSD), Solvent Accessible Surface Area (SASA) and Ramachandran plot analysis. It was observed that STI has an unusual β-sheet structural stability under the influence of oscillating electric fields. Ramachandran plots were also used to analyze the stability of the molecules obtained on treatment with temperatures (300 K to 393 K) and oscillating electric fields (0.5 V/nm at 2.45 GHz).

The experimental results showed that the *in-vitro* protein digestibility increased with increase in time and temperature during microwave processing and conventional thermal processing. Similarly reduced trypsin inhibitor activity (TIA) values were obtained with an increase in time and temperature of the processing conditions. Therefore, microwave processing can be used as a potential alternative method of processing to improve the overall quality of soymilk. MD simulations have been introduced as a tool to visualize the changes in the stability of secondary protein structures during various processing conditions of time and temperature. The studies also established the relation between various processing methods and conformations of secondary structure of proteins. The solvent accessible area of cysteine residues increased with an increase in the temperature (300 K to 394 K), both in the presence and absence of the oscillating electric field, influencing the disulphide bridge stability. The results showed that the core tryptophan residues of the STI protein increased significantly by changing the conformation of the secondary structure during conventional heating and microwave processing, thus affecting the functional properties of the protein.

## RÉSUMÉ

Les techniques de traitement et conservation des denrées périssables permettent de fabriquer des aliments nutritif et salubre, et d'augmenter la durée de conservation des produits alimentaires. Certaines de ces techniques permettent d'accroître la digestibilité des aliments, entraînant une plus grande biodisponibilité des nutriments et l'élimination de facteurs indésirables. Bien que plusieurs études ont été effectuées pour établir la validité de diverses méthodes de traitement comme la stérilisation, la pasteurisation, l'extrusion, l'homogénéisation, le blanchiment, l'injection de vapeur, les ultra-hautes températures (UHT), l'utilisation combinée de hautes températures et pressions, le chauffage ohmique et les traitements à très haute pressions (HPP), on constate que l'état de la connaissance sur les effets de la transformation au niveau atomique et moléculaire des aliments est limité. Une meilleure compréhension des changements au niveau moléculaire causés par les procédés de transformation est nécessaire pour développer de nouveaux procédés industriels pour la fabrication d'aliments nutritifs, savoureux et sécuritaires. Dans cette étude, les effets des traitements thermiques conventionnels et par micro-ondes sur des échantillons de lait de soja brut pour l'inactivation des inhibiteurs de la trypsine ont été évalués. Des essais sur la digestibilité in-vitro des protéines (IVPD) ont été menés pour mieux comprendre les effets de ces procédés sur les protéines de lait de soja. Les traitements par micro-ondes ($70^0$C, $85^0$C et $100^0$C pour 2, 5, et 8 min) ont été comparés aux traitements thermiques conventionnels ($70^0$C, $85^0$C et $100^0$C pour 10, 20, et 30 min) et optimisés en utilisant la méthodologie des surfaces de réponses (RSM).

Des techniques de simulation de la dynamique moléculaire (MD) ont été utilisées pour étudier et visualiser le comportement des protéines exposées à des stresses externes causés par les champs micro-ondes, les températures élevées et les durées de traitement. La protéine de soya (STI) qui

agit comme inhibiteur de l'activité enzymatique de la trypsine et de la chymotrypsine a été utilisée pour les simulations MD. Lors de ces simulations, l'intensité du champ électromagnétique était de 0,5 V/nm et la fréquence de 2450 MHz. Cette simulation a permis d'avoir un aperçu de l'effet du champ électromagnétique sur la conformation des protéines. Ces effets ont été évalués et comparés par le biais des déviations de l'écart quadratique moyen (RMSD), les surfaces de la zone accessible aux solvants (SASA) et les tracés de Ramachandran. Il a été observé que sous l'influence des champs électriques oscillants les noyaux résiduels de tryptophane du STI avaient une stabilité structurelle de β-feuille inhabituelle. Les tracés de Ramachandran ont également été utilisés pour analyser la stabilité des molécules obtenues après le traitement à des températures allant de 300 à 393 K et exposées aux microondes.

Les résultats ont démontré que la digestibilité in-vitro des protéines augmentait avec l'augmentation du temps et de la température de traitement (micro-ondes et thermique conventionnel). De plus, l'activité d'inhibiteur de trypsine (AIT) a diminué avec l'augmentation du temps et de la température des traitements. Le sur-traitement thermique conventionnel requis pour la désactivation des inhibiteurs de trypsine a endommagé d'autres protéines du soya et du fait réduit sa qualité nutritive. Du fait, cette étude a démontré que le traitement par micro-ondes du lait de soya permettaient de fabriquer un lait de meilleur qualité. De plus, il a été observé que les surfaces de la zone accessible aux solvants des résidus de cystéine augmentaient avec l'augmentation de la température (300 K à 394K), à la fois en présence et en l'absence du champ électrique oscillant, et influencé la stabilité des ponts disulfure. On a trouvé que les résidus de noyaux de tryptophane de la protéine STI avaient augmenté de manière significative en modifiant la conformation de la structure secondaire, lors du traitement thermique conventionnel et par traitement de micro-ondes, ce qui a altéré les propriétés fonctionnelles de la protéine.

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## Thesis Format

This thesis is submitted in the format of papers suitable for journal publication. This thesis format has been approved by the Faculty of Graduate and Postdoctoral Studies, McGill University, and follows the conditions outlined in the Guidelines: Concerning Thesis Preparation, which are as follows:

**“**As an alternative to the traditional thesis format, the dissertation can consist of a collection of papers of which the student is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research. The structure for the manuscript-based thesis must conform to the following:

1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis).

2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.

3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts.

The thesis must include the following

(a) A table of contents;

(b) An abstract in English and French;

(c) An introduction which clearly states the rational and objectives of the research;

(d) A comprehensive review of the literature (in addition to that covered in the introduction to each paper);

(e) A final conclusion and summary;

4. As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

5. In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers”.

# Contribution of Authors

The following are the manuscripts prepared for publication:

1. **Harish Vagadia, Brinda**; Vanga, Sai Kranthi; Raghavan, Vijaya. 2015. "Inactivation methods of Soybean Trypsin Inhibitor- a Review" (Under Review-2nd Revision)
2. **Harish Vagadia, Brinda**; Vanga, Sai Kranthi; Singh, Ashutosh; Raghavan, Vijaya. 2016. "Effect of Thermal and Electric Fields on Soybean Trypsin Inhibitor- a molecular modelling study" (Accepted)
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The work reported here was performed by Brinda Harish Vagadia and supervised by Dr. Vijaya Raghavan of the Department of Bioresource Engineering, Macdonald Campus of McGill University, Montreal. The entire research work was carried out at the Postharvest Technology laboratory, Macdonald Campus of McGill University, Montreal.

Dr. Raghavan has also provided scientific advice and is directly associated with editing and reviewing the manuscript. Dr. Ashutosh Singh has provided the technical help with regard to the molecular modeling software GROMACS, data analysis (Response Surface Methodology) and also participated in reviewing the manuscript. Mr. Yvan Gariepy is an academic associate in the Department of Bioresource Engineering and is actively involved in providing the research facilities for conducting the experiments. Sai Kranthi Vanga has provided technical support for the research work and also contributed in reviewing the manuscript for publication.

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# CHAPTER I

# INTRODUCTION

Global food security is one of the greatest challenges faced by mankind today, apart from settling the issues of global warming. Technological breakthroughs is necessary to cater the needs of food, increasing incomes and urbanization, by 2050, for a global population of 9.6 billion. In order to overcome the challenges of food security such as malnutrition, obesity and micronutrient deficiencies, researchers and industrialists must address the food system activities of production from crop, livestock, trees, freshwater and marine sources; processing; environmental, health and social objectives; and availability and access to the population (Pinstrup-Andersen, Pandya-Lorch, & Rosegrant, 2001).

Soybeans are a globally important crop, providing oil and protein. They have been first traced in China, in the early eleventh century B.C. Soybeans have been regarded as an important protein source for over five thousand years. This crop has been introduced to the Western countries since the 20$^{th}$ century. In Canada, soybeans were first cultivated in Ontario in the year 1881 and since then, it has been one of the most grain legume crop in the country. The demand for soybean products have been continuously increasing over the past few decades (Vijaya Raghavan, Harper, & Kienholz, 1974). Soybeans are an exceptional source of vital nutrients such as dietary fiber, manganese, iron and several B vitamins. They are considered as a source of complete protein, containing significant amounts of essential amino acids to the human body (Henkel, 2000). The diverse products derived from soybean are soybean sprouts and nuts, soybean flours, soybean oil and meals, soymilk, tofu, okara, tempeh, soy sauce, nutritional supplements, non-dairy desserts, soy yogurt and texturized meat alternatives.

Soymilk is the most consumed soy product all over the world. An 8-ounce glass of plain soymilk contains 10 grams of protein. The rise in popularity of soymilk can be attributed to the fact that it addresses the needs of customers with various health issues, including lactose intolerance, milk allergy, galactosemia, veganism and also for customers concerned with antibiotics or growth hormones present in cow's milk. As the soymilk industry expands, the producers are expected to invest in a greater research to develop newer varieties with better taste and nutritional quality (Kerwin, 2004; Spelbrink, Gerrits, Mooij, & Giuseppin, 2011). One of the main problems, the soymilk industry faces is the elimination of anti-nutritional compounds present in soy products that interfere with the flavour and nutritional quality. The various anti-nutritional factors present in soy products are lipoxygenase, urease, protease trypsin inhibitors (TI's), lectins, tannins, oligosaccharides, saponins, alkaloids, phenolics, phytic acid and isoflavones. Lipoxygenase is responsible for the undesirable flavour formed by oxidation of polyunsaturated fatty acids. The protein utilization and digestion in the body is affected by the ingestion of trypsin inhibitors (Francis, Makkar, & Becker, 2001; Prachayawarakorn, Prachayawasin, & Soponronnarit, 2006).

Soybeans are the most concentrated source of TI's among common food and feed products. Raw soybeans have been reported to contain 20-127 mg/g protein of trypsin inhibitors (mostly Kunitz type soybean trypsin inhibitor) (Anderson & Wolf, 1995). The adverse effects of TIs continue to be a major concern. Currently, since there are no regulatory upper safe limits for dietary TI in foods, there is no guarantee that commercial soybean products have been processed to a point where the effects of TI cannot be seen. For instance, 28% of the original trypsin inhibitor activity was retained in a soy infant formula. Similarly, several commercial soya beverages have been reported to maintain up to 70% TIs of that in raw soybean, which could greatly hinder the activity

of digestive enzymes trypsin and chymotrypsin in the human body (Gilani, Xiao, & Cockell, 2012).

### 1.1 Hypothesis and implications

Extensive research has been done to inactivate TI's by heat processing such as autoclaving, steam processing, boiling, infrared, and extrusion techniques, but prolonged heating may damage the overall protein quality (Liener, 1981; Stewart, Raghavan, Orsat, & Golden, 2003). Studies on the inactivation of soybean trypsin inhibitor in soymilk using microwave processing is limited. This can be regarded as a potential alternative to existing conventional processing methods in food industry for inactivation of anti-nutritional factors. Also, the relation between various processing methods and conformation of secondary structure of proteins has been established Molecular Dynamics (MD) simulations have been introduced as a tool to visualize these changes in the secondary structures during various processing conditions.

Thus, in this study, the effect of microwave and thermal treatment on digestibility and trypsin inhibitor actvity of soymilk were determined. And also, their conformational secondary structure changes were visualized using MD simulations.

### 1.2 Objectives

#### 1.2.1 Overall Objective

The overall objective is to study the effects of microwave processing on soymilk protein, in order to maximize its digestibility and minimize the trypsin inhibitor activity. The changes in the protein structure of soybean trypsin inhibitor during various process simulations were evaluated using molecular simulation as a visualization tool for understanding its behaviour at molecular level.

### 1.2.2 Specific Objectives

1. To investigate and optimize the suitable conditions of time and temperature during microwave processing to obtain soymilk with increased digestibility and inactivate trypsin inhibitors, in comparison to the conventional thermal treatment.
2. To evaluate the effects of thermal and oscillating electromagnetic fields generated by molecular dynamic simulations on the stability of Soybean trypsin inhibitor protein.

## CHAPTER II

## INACTIVATION METHODS OF SOYBEAN TRYPSIN INHIBITIOR – A REVIEW

### 2.1 ABSTRACT

Soybeans are an essential source of low cost protein. They are widely consumed due to their functionality and nutritive value. Recently, the consumption of soybeans has been increasing due to its beneficial effects on human health such as prevention and treatment of various chronic illnesses which include cardiovascular diseases and various forms of cancer. However, they contain a variety of bioactive anti-nutritional compounds including protease trypsin inhibitors, phytic acid and isoflavones that exhibit undesirable physiological effects and impede their nutritional quality. Inactivation of these trypsin inhibitors, along with deleterious enzymes, microbes, bioactive components and increasing the protein quality by improving its texture, colour, flavour, functionality and digestibility are the most important factors to be considered in crucial stage in manufacturing of soy products. This review discusses the principal methods (physical and chemical) adopted by food researchers and industrialists to improve the nutritional and functional properties of soybeans and to eliminate the trypsin inhibitors present in them. Discussions also includes the innovative ways of using molecular modelling simulations for studying proteins and enzymes.

**Keywords:**

Soybean trypsin inhibitors; molecular modelling; physical treatments; thermal; chemical processing

## 2.2 INTRODUCTION

Proteins are one of the principal components in the diet essential for the survival of the organisms. They are responsible in supplying adequate amounts of amino acids to the body. The availability of amino acids and proteins to the human body for nutrition depends on various factors like source of the protein, prior protein processing treatments, and interactions with the other components of food, their digestibility, absorption and utilization in the human body. Soybean (*Glycine max*), is one of the vital and widely consumed legume crops in the world. Soybean crops have an economic impact of $114 billion around the world. Figure 2.1 represents the global soybean production for the year 2013-2014. USA and Brazil are the leading producers of soybean in the world (He & Chen, 2013).

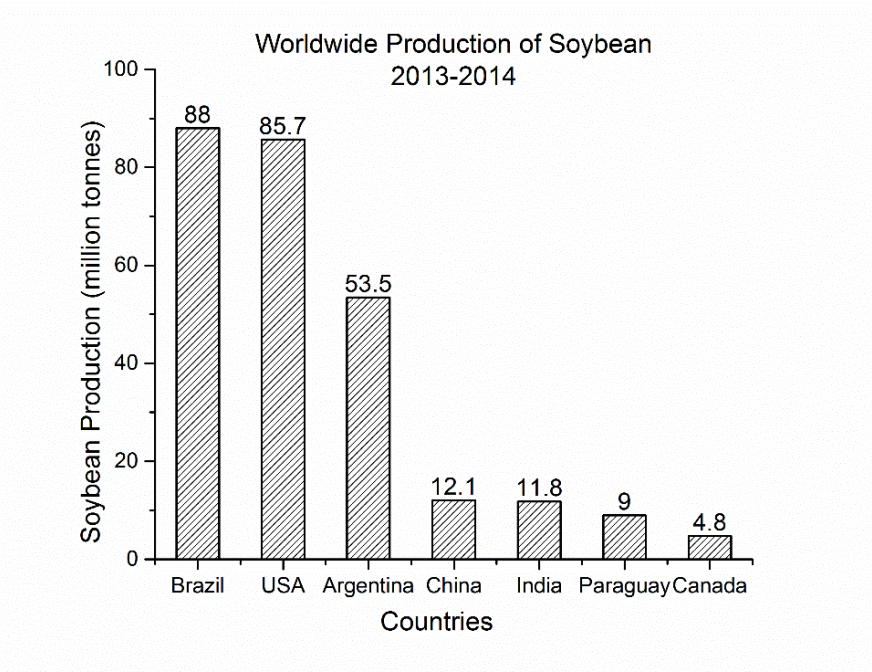



**Figure 2.1** Global Soybean Production, Source: USDA Database

**Table 2.1** Nutritional profile of raw soybean (value per 100 g)

| COMPONENTS | MASS |
|---|---|
| **Carbohydrates** | **30.0g** |
| Sugars | 7.3g |
| Dietary Fiber | 9.3g |
| **Fat** | **19.9g** |
| Saturated | 2.8g |
| Monounsaturated | 4.5g |
| Polyunsaturated | 11.2g |
| **Protein** | **36.5g** |
| **Minerals** | |
| Calcium | 28% (277 mg) |
| Iron | 126% (15.70 mg) |
| Phosphorus | 101% (704 mg) |
| Potassium | 38% (1797 mg) |
| Magnesium | 76% (280 mg) |
| Sodium | 0% |
| Zinc | 49% (4.89 mg) |
| **Vitamin** | |
| Vitamin B6 | 29% (0.3 mg) |
| Vitamin C | 10% (6.0 mg) |
| Vitamin K | 45% (0.04 mg) |
| **Water** | **8.5g** |

Source: USDA Nutrient Database

According to the USDA nutritional database, soybean seeds consists about 36.5% protein, 19.9% lipids, 30% carbohydrates and 9.3% dietary fibre. The nutritional profile of soybeans is summarized in Table 2.1, the percentages mentioned are relative to US recommendations for adults. Soybeans produce more protein and oil per unit than almost any other leguminous crop. Soybean oil is the second largest vegetable oil produced in the world. It contains 15.6% total saturated fatty acids, 57.7% total polyunsaturated fatty acids and 22.8% total monosaturated fatty acids (U.S Food & Drug Administration, 2012). After conducting a thorough investigation following the guidelines adopted by the U.S Food and Drug Administration (FDA) and World

Health Organization (WHO) for protein assessment, the protein quality of soybeans is rated along with milk and meat proteins. It has a Protein Digestibility Corrected Amino Acid Score (PDCAAS) of 1, which is the highest for any legume, indicating that soy protein provides most of the amino acids to human nutrition (Hoffman & Falvo, 2004). Their nutritional composition varies depending on variety, location, climate and farming practises followed. They are also a major source of oligosaccharides, omega 3-fatty acids, vitamins, and minerals. Soy proteins have been widely used to formulate foods with a goal of improving their nutritional and functional qualities due to the high protein level and well-balanced amino acid composition (Aijie, Shouwei, & Li, 2014). The amino acid composition of soybean storage proteins is indicated in Table 2.2

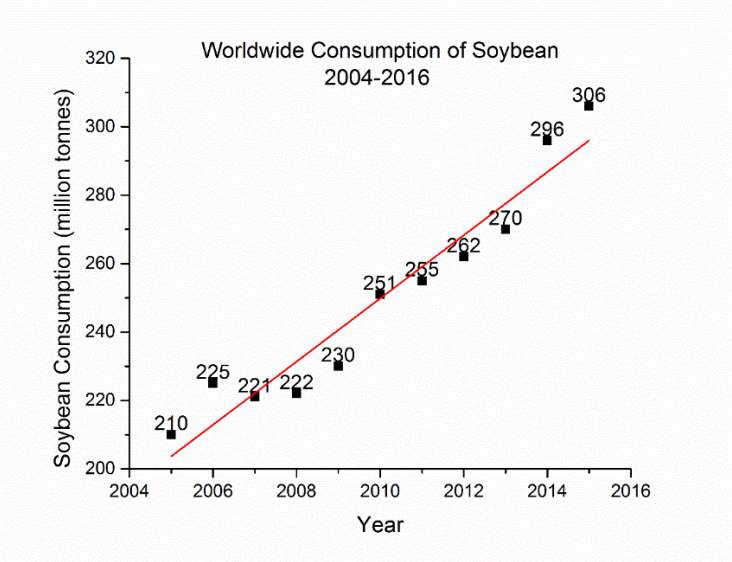
**Table 2:** Amino acid composition of soybean seed

| Amino Acid | mg/g protein |
|---|---|
| Arginine | 77.16 (7.71%) |
| Alanine | 40.23 (4.02%) |
| Aspartic acid | 68.86 (6.88%) |
| Cysteine | 25.00 (2.50%) |
| Glutamic acid | 190.1 (19.01%) |
| Isoleucine | 51.58 (5.15%) |
| Leucine | 81.69 (8.16%) |
| Lysine | 68.37 (6.83%) |
| Phenylalanine | 56.29 (5.62%) |
| Proline | 52.91 (5.29%) |
| Serine | 54.05 (5.40%) |

Source: (Asif & Acharya, 2013; Liu, 1997)

The consumption of soybean has been increasing over the past few years due to its positive effects on human health (He, 2013). Further, it acts as a main protein source for people following vegan diet around the world. Figure 2.2 indicates the rise in worldwide soybean consumption over the past 12 years.

**Figure 2.2 Soybean Consumption 2004-2016 (million tonnes)**




Source: USDA Database

The commercial products derived from soybean are very diverse and include soybean sprouts and nuts, soybean flours, soybean oil and meals, soymilk, tofu, okara, tempeh, soy sauce, nutritional supplements, non-dairy desserts, soy yogurt and texturized meat alternatives (Spelbrink, Gerrits, Mooij, & Giuseppin, 2011). Epidemiological studies suggest that consumption of soybeans plays an essential role in prevention and treatment of various chronic illnesses including cardiovascular diseases, lowering of plasma cholesterol, protection against bowel and kidney diseases and osteoporosis. In addition to them, there were also reports on consumption of soybean reducing the risk of breast, prostate and colon cancers, diabetes, and obesity (Friedman & Brandon, 2001; Wiseman, Andersen, & Markham, 2006).

However, in spite of numerous favorable nutritional characteristics, soybean consists of a variety of anti-nutritional bioactive compounds that exhibit undesirable physiological effects impeding

their nutrient uptake as shown in (Table 2.3). These anti-nutritional compounds are broadly classified as lipoxygenase, urease, protease trypsin inhibitors, lectin, and the others in smaller quantities such as tannins, oligosaccharides, saponins, alkaloids, phenolics, phytic acid and isoflavones. Isoflavones are known as phytoestrogen; they are plant compounds that have estrogen-like structures. The application of soybean in animal and human nutrition is hindered by the presence of the above mentioned anti-nutritional factors in them (Martín-Cabrejas, et al., 2009).

**Table 2.3** Anti-nutritional Factors in Soybeans

| Heat Liable | Heat Stable |
|---|---|
| Trypsin inhibitors | Saponins |
| Antivitamins | Estrogens |
| Phytates | Allergens |
| Hemagglutinins | Flatulence factors |

Adopted from (Liener, 1981; Martín-Cabrejas, et al., 2009)

Raw soybeans cannot be used as human food or animal feed, due to the presence of the anti-nutritional factors that harm the consumer. In order to improve the retention and utilization of soy protein and minerals in the digestive tract, inactivation of the anti-nutritional factors is a must which makes it a major issue for the food processors and researchers (Liu, 1997). Studies conducted by Raghavan *et al.*,(1974) showed a direct association between processing and the protein availability when soybean meal was used as chicken feed. They reported that processed soybean meal can affect the protein intake and in turn improve the weight gained by the chicken over a course of time. They predicted that denaturation of anti-nutritional factors is one of the major reasons for this weight gain (Vijaya Raghavan, Harper, & Kienholz, 1974).

The negative impacts due to the ingestion of these anti-nutritional factors have also been reported. For instance, phytates are mineral chelating agents, thereby hinder mineral uptake. Urease

decomposes urea to ammonia and carbon dioxide whereas lipoxygenase is responsible for the undesirable flavour formed by oxidation of polyunsaturated fatty acids (Francis, Makkar, & Becker, 2001; Iassonova, Johnson, Hammond, & Beattie, 2009; Prachayawarakorn, Prachayawasin, & Soponronnarit, 2006). Among all the antinutrients present in wide variety of foods, trypsin inhibitors are of high importance as they affect the protein utilization and its digestion. They are commonly present in various crops, nuts and vegetables such as soybean, rice bran, corn, black gram, faba beans, chickpea, mung bean, pigeon pea, sesame seeds, potatoes, kidney beans, lima beans, lentils, peanuts, wheat, winged bean, cowpea, barley, cucumber, pumpkin and velvet beans. They are also present in animal feed crops such as Barbados nuts (*Jatropha curcas)*, Moringa (*Moringa oleifera*) and castor oil plants (Makkar, Siddhuraju, & Becker, 2007). Soybean is one of the legume that accounts for the highest amount of protease inhibitors, about 2% of soybean meal or 2-6% of soybean protein (Saini, 1989; Vinh & Dworschák, 1986; Xiao, Wood, Robertson, & Gilani, 2012). The trypsin inhibitor content in raw soybean products is summarized in Table 2.4.

**Table 2.4** Trypsin Inhibitor Activity(mg/g) of raw soy products (measured by their ability to inhibit pure trypsin)

| Sample | TIA (mg/g sample) |
|---|---|
| Whole soybeans | 16-27 |
| Raw flour | 28-32 |
| Roasted soy flour | 8-10 |
| Soy concentrate | 6-8 |
| Soy protein isolate | 1-30 |
| Raw defatted flour | 28-32 |
| Dehydrated soymilk | 11-14 |

Adopted from(Anderson & Wolf, 1995; Baintner, 1981; He & Chen, 2013; Huang, Kwok, & Liang, 2008; Kwok, Qin, & Tsang, 1993; Li & Yu, 2012; Makkar, Siddhuraju, & Becker, 2007)

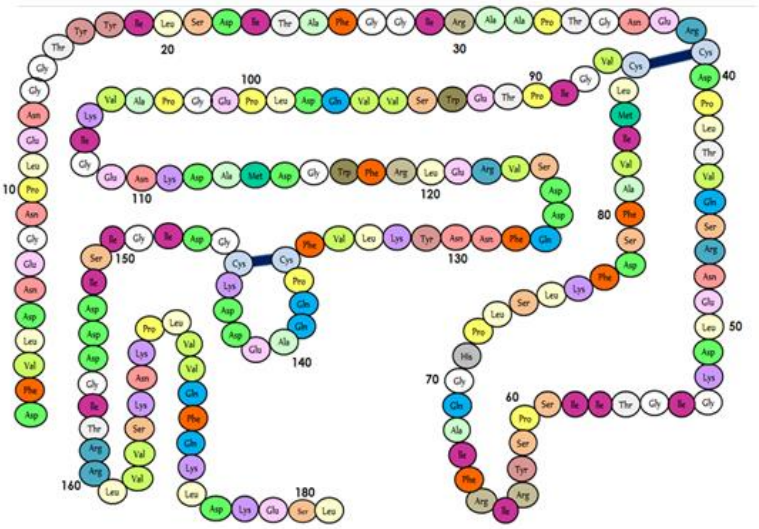
The consumption of trypsin inhibitors can cause pancreatic hypertrophy or hyperplasia (an increase in the acinar cells of the pancreas) in the body (Embaby, 2010). The normal pancreatic functions are altered, leading to an increase in the secretory activity of pancreas. The pancreas are controlled by negative feedback mechanism, enzyme secretion is inversely related to level of trypsin in the gut. Hence there is an over production of digestive enzymes trypsin, chymotrypsin and elastase in the body. It has been reported that increase in the size of the acinar cells leads to pancreatic enlargement and induce pancreatic tumours, in experimental rats. Also, in consequence to amino acids secreted in excess, there are reports of growth suppression in rats. Therefore the normal process of digestion is deviated due to the ingestion of trypsin inhibitors (Cheftel, Cuq, & Lorient, 1985; Palacios, et al., 2004; Embaby, 2010; I. E. Liener, 1994; Makkar, et al., 2007).

Trypsin inhibitors are a group of serine protease enzymes. They reduce the biological activity of the digestive enzymes trypsin and chymotrypsin. Trypsin is a proteolytic enzyme which is important for the digestion of proteins in living organisms. It is a globular protein with a molecular weight of 24-kDa with 220 residues. This enzyme protein is produced in its inactive form called 'trypsinogen' in the pancreas which is then activated during digestion as it enters the small intestine (Walsh, Kauffman, Kumar, & Neurath, 1964). The ingestion of trypsin inhibitors can result in the formation of an irreversible trypsin enzyme- trypsin inhibitor complex which leads to a trypsin enzyme drop in the intestine, interfering in protein digestibility process (Cabrera-Orozco, Jiménez-Martínez, & Dávila-Ortiz, 2013). Thus, presence of trypsin inhibitor directly affects the digestibility of the soybean consumed.

Various researchers have evaluated the digestibility of soybean protein using *in vitro* protein digestibility (IVPD) methods. Su et al., (2002) evaluated the trypsin inhibitor activity (TIA) and IVPD in both soybean and soymilk. TIA was estimated to be 143 and 106.5 TIU/g soybean in soybean and soymilk samples respectively (Su & Chang, 2002). The IVPD was found to be around 75% for raw soybean and soy milk. The digestibility evaluated by Shin et al., (2013) was also in the same range between 74.4% - 77.1% except the steamed soy flour whose digestibility was recorded to be 88.7% (Shin, Kim, & Kim, 2013). The soybean protein isolate (SPI) produced by separating the fat out, has showed an increased IVPD compared to whole soybean. The IVPD values were estimated to be 79% - 84% depending on the storage temperature (Pinto, Lajolo, & Genovese, 2005). It has to be noted that the digestibility values of soybean are much lower compared to peanuts whose IVPD values are over 85% (Vanga, Singh, Kalkan, et al., 2015). Liener et al., (1970) have tabulated the digestibility values of various protein sources including peanut, soybean and animal protein. It can be clearly observed that the IVPD values recorded for soybean are much lower compared to other sources. This is due to the presence of trypsin inhibitors and other anti-nutrients present in soybean. Thus, their denaturation will directly result in increased digestibility (Liener, 1970; Kumar, Rani, Pandey, & Chauhan, 2006).

**Figure 2.3** Primary and tridimensional structure of Soybean Kunitz Trypsin Inhibitor (SKTI)

A) Primary Structure of Kunitz trypsin inhibitor from soybean



Reproduced with permission from (Cabrera-Orozco et al., 2013). The disulfide bonds shown in solid blue lines.



B) Tridimensional Structure of Kunitz type inhibitor from soybean

PDB CODE- 1AVU

Adopted from (Song & Suh., 1998b)

The Soybean kunitz trypsin inhibitor (SKTI) is a small, stable monomeric, non-glycosylated, globulin type protein present in the soybean seeds. SKTI protein consists of 181 amino acid residues with a molecular weight of 21.5 kDa and an isoelectric point at pH 4.5 (Kunitz, 1947). They act as storage proteins which protects the plant against microbial proteinases and also to regulate the endogenous proteinases in plants; but they have an unusual amino acid composition resulting in their inhibitory property upon consumption. The overall structure of SKTI is spherical with a diameter of 3-5 nm consisting of 12 criss-cross antiparallel β strands, stabilized by hydrophobic side chains as shown in figure 2.3 (Song & Suh, 1998). It is resistant to thermal, chemical and enzymatic (proteolysis by pepsin) denaturation in a gastric environment (Astwood, Leach, & Fuchs, 1996). SKTI has two disulfide bridges, Cys 39-Cys 86 and Cys 136-Cys 145, as shown in figure 2.3 highlighting the tridimensional and primary structure of Kunitz Trypsin Inhibitor (KTI) from soybean. Therefore, in order to inactivate the trypsin inhibitors, the two disulfide bonds present in them have to be cleaved. This can be achieved by various processing methods which includes physical, chemical and enzymatic treatments at different conditions of temperature and time (Birk, 1985; Blow, Janin, & Sweet, 1974; Chen, 2015; Friedman & Brandon, 2001; Van Der Ven, Matser, & Van Den Berg, 2005).

Overall, the purpose of this review is to highlight various methods adopted as mentioned above by the food industry and food researchers to increase and improve the nutritional and functional properties of soybean by reducing the trypsin inhibitor activity.

## 2.3 PROCESSING METHODS FOR INACTIVATION OF SOYBEAN TRYPSIN INHIBITOR

An optimal process is the one that inactivates the trypsin inhibitors, along with deleterious enzymes, microbes, biological active components while increasing its protein quality by improving

its texture, colour, flavour, functionality, digestibility and retention of nutrients. The nutritional, physiochemical and functional properties of soybean and soybean products can be altered using physical and chemical treatments. These treatments mainly include heating, pH adjustment, hydrolysis, high pressure, PUV, PEF and covalent attachment of other constituents. For utilizing soybeans in food and feed industries, it is most important to select an appropriate method for treating raw soybean (Vanga, Singh, & Raghavan, 2015b).

### 2.3.1 Physical Treatments

Soybean proteins can be resistant to digestion until their internal chemical structure is changed under the influence of high temperatures or mechanical stress when compared to various other protein sources. Most treatments involve heating the soybean samples for a certain amount of time. The heat energy usually breaks the physical arrangement of the molecules making it easier for enzyme digestion. Thermal treatment for the destruction of SKTI is influenced by temperature, moisture content and the treatment duration (Zilic, Bozovic, & Sukalovic, 2012). Physical treatment is the classical approach to inactivate trypsin inhibitors. The only disadvantage if not optimised carefully is it can indiscriminately destroy other essential nutrients present in the legume (Agrahar-Murugkar & Jha, 2010; Chen, 2015; Jasti, Lavanya, & Fadnavis, 2015; Murugkar, 2015).

#### 2.3.1.1 Conventional treatments

Thermal inactivation of trypsin inhibitors depends on duration time, temperature and moisture content of the samples. Forced conventional drying and roasting are some common heat treatment methods used for the elimination of trypsin inhibitors (Carvalho, et al., 2013; Stewart, Raghavan, Orsat, & Golden, 2003). Roasting mainly includes the use of rotary drum dryer, salt-bed roasting

and conventional grain dryer at temperatures varying between 110-170$^{o}$C, which can inactivate the TIA up to 85%, simultaneously drying the soybeans to an optimal moisture content for storage. Thermal treatment in the oven at 200$^{0}$C for 20 min, significantly reduced the activity of trypsin inhibitors in whole soybean flour (Andrade, Mandarino, Kurozawa, & Ida, 2016). But one of the major disadvantages of the above mentioned method is non-uniform cooking and drying with uneven temperature distribution, especially when the drying bed thickness is uneven (Prachayawarakorn, et al., 2006).

Conventional hot air drying at 100$^{o}$C for two hours reduced the trypsin inhibitor activity to safe limits. The reduction was up to the required 80%, without affecting the nitrogen solubility and colour of the soy flour. In the same experiment, steaming (10 min) of soybean flour followed by hot air drying at 60$^{o}$C reduced the inhibitor activity levels to 80% after three hours (Agrahar-Murugkar & Jha, 2010).

According to Kaur et al.,(2012) the most effective treatment for the inactivation of the bioactive compounds such as trypsin inhibitors in cereal bran is by use of dry heat or moist heat (Kaur, Sharma, Dar, & Singh, 2012). To improve the protein digestibility and to reduce the processing time required for the inactivation of anti-nutritional factors, soaking of soybeans at ambient temperature as a pre-treatment is crucial. It is observed that soaking can effectively remove the protease inhibitors and other undesirable factors such as indigestible sugars into the water, thereby reducing the cooking time by 70%. Soaking soybeans in water overnight followed by heat treatment resulted in an increase in the overall inactivation by 40% when compared to the unsoaked beans (Bayram, Kaya, & Öner, 2004). In the case of cowpea, soaking along with steam-blanching resulted in higher inactivation of trypsin inhibitor than water blanching without any loss of nutrients (Wang, Lewis, Brennan, & Westby, 1997).

Germinated soybeans are an excellent source of riboflavin and ascorbic acid. The activity of the proteolytic enzymes is increased during germination of legumes (Wang et al., 1997). Studies have shown that germination of soybeans reduces the trypsin inhibitor activity and also increased the *in-vitro* digestibility of soybean protein (Savelkoul, Van der Poel, & Tamminga, 1992). From the results of Mostafa et al., (1987), we can conclude that the TIA can be reduced by 32% by 6 day germination process which was followed by a 24 h soaking process with distilled water. The inactivation was faster at higher germination temperature. Residual trypsin inhibitor activity was decreased by 68% continuously during germination (at 100% RH) for 144 h at $35^0$C (Mostafa, Rahma, & Rady, 1987; Kumar, Rani, Pandey, & Chauhan, 2006).

According to Osman et al., (2002) a 96% reduction in trypsin inhibitor activity was seen, when the soybeans were autoclaved at $121^0$C for 60 min (Osman, Reid, & Weber, 2002). In case of chickpeas, Alajaji et al., (2006) reported there was a decrease in anti-nutritional factors based on the cooking treatment. Reductions of 83.2% and 82.2% were seen in autoclaving ($121^0$C, 35 min) and boiling ($100^0$C, 90 min) respectively (Alajaji & El-Adawy, 2006). Further studies by Carvalho & Sgarbieri, suggeted that autoclaving of prior 12 h soaked whole soybeans and flour ($121^0$C, 5 min) resulted in 55% trypsin inhibitor inactivation and 75% in whole soybeans whereas boiling for 30 min at a pH of 12 completely inactivated the anti-nutritional trypsin inhibitors. Further autoclaving for 20 min at same conditions, resulted in 65% inactivation of trypsin inhibitors in soy flour and 80% in whole (Carvalho & Sgarbieri, 1997). From the above mentioned studies, it is quite clear that autoclaving can be an effective process for inactivating the trypsin inhibitor enzyme in soybean and soaking reduces the processing time conditions of the treatment. Similar conclusions were made by Kwok *et al.*, in which they mentioned that destruction of trypsin

inhibitors are temperature dependent ($100^0$C, 40 min or $125^0$C, 5 min) (Kwok, Liang, & Niranjan, 2002).

Ultra-high temperature process (UHT) and steam injection have been used in the large scale processing plants of soy products for the inactivation of anti-nutritional factors. Soymilk processed by traditional batch stove cooking and steam injection ($100^0$C for 20 min), blanching methods (70-$85^0$C, 30 seconds -7.5 min) and UHT ($135^0$C - $150^0$C, 10-50 seconds) have residual trypsin inhibitory levels of 13%, 50% and 10 % , proving UHT methods to be most efficient (Yuan & Chang, 2010). Kowk *et al.,* (Guerrero-Beltrán, et al., 2009; K. Kwok, Qin, & Tsang, 1993) recommended that in case of soymilk, trypsin inhibitors can be inactivated to 10% at temperatures below $100^0$C. This can be achieved by preheating the soymilk ($80^0$C for 2 min) prior to ultra- high temperature treatment. Further, this will also aid in reducing the damage to the nutrients (like lysine, sulfur amino acids and vitamins) that are caused at high temperatures. Savage, Wei, Sutherland, & Schmidt, (1995) reported an 80% inactivation of trypsin inhibitors by the process of blanching soybeans at $100^0$C for 12 min. A reduction of 20% of trypsin inhibitors were seen at High Temperature Short Time (HTST) ($137^0$C at 77 seconds) of soymilk compared to the conventional batch boiling process (15 min) whose results were assayed by ELISA technique (Rouhana, Adler-Nissen, Cogan, & FrØKiÆR, 1996).

#### 2.3.1.2 Novel treatments

Ohmic heating is an advanced thermal processing method wherein an electric current is passed through food (Wang, et al., 2007). Electrochemical effects of ohmic heating are capable of deactivating trypsin inhibitors. During this treatment, the inactivation of trypsin inhibitors is dependent on the electric voltage. According to the studies conducted by Lu *et al.,* (2007). Ohmic

heating (220V, 50Hz) for periods over 3 min efficiently inactivates TI when compared to induction cooker or electric stove methods over 3 min. The residual trypsin inhibitory activity was 13% (Ohmic heating) which is significantly lower compared to 19% in induction cooker and electric stove. Power of induction cooker were used to simulate the same conditions as ohmic heating (220V, 50Hz) (Lu, et al., 2015). Soymilk processed by this method also yields high quality tofu when compared to other conventional heating procedures (Li & Toyoda, 2011; Wang, et al., 2007)

Various studies presented in the literature on wide variety of legumes indicate that industrial scale dielectric heat treatment technology at 42 MHz (Radio frequency) and 2450 MHz (Microwave) were found to have a beneficial effect on the overall quality of the protein. Microwave heating is considerably effective in the inactivation of the protease inhibitors in selected legume seeds without affecting the nutrient and protein quality. Soaking prior to microwave treatment of legumes significantly reduced the activity of trypsin inhibitors by a greater extent compared to the microwave heated dry method. This is due to the higher moisture content in the soaked soybeans which could have resulted in better dielectric properties. TIA of legume seeds such as faba beans, chickpeas, soybeans, lentils and common beans showed same degree of inactivation by microwave heating method at 2450 MHz at power 0.54 kW compared to conventional heating treatment i.e. boiling followed by hot air treatment (60$^{o}$C for 20 hours) (Hernandez-Infante, Sousa, Montalvo, & Tena, 1998). According to Jourdan *et al.,* TIA can be reduced to 3% using microwave treatment at 2450 MHz for 15 mins in case of Brazilian beans (Jourdan, Noreña, & Brandelli, 2007). Microwave treatment (10 min) at 2450 MHz has also been successful in eliminating anti-nutritional factors in soaked velvet beans overnight to 8% residual TIA compared to the same treatment (12 min) for unsoaked velvet beans (Kala & Mohan, 2012). Trypsin inhibitors present in winged bean seeds were completely destroyed by microwave heating (3-5 minutes, 2450 MHz)

(Esaka, Suzuki, & Kubota, 1987). According to (Zhong, Wang, & Zhao, 2015a), dielectric heating was more efficient in the inactivation of anti-nutritional factors compared to elevated temperature and pressure treatments. Black soybean samples were tested in this process and it was found that the TIA was eliminated after a 30 min microwave treatment of 2450MHz. Large amounts of soybean (45 kg in 30 cm layers) when treated at $120^0$C by microwave, have reported an inactivation of 93% soybean trypsin inhibitors (Petres, et al., 1990). It is clear that microwave treatment reduces the processing time required to achieve safe level of inactivation when compared to conventional methods. In a study conducted by Barac *et al.*, the trypsin inhibitor activity (TIA) was reduced to 13.33% by microwave roasting at 2450 MHz for 2 minutes in soybean. The treatment also had a strong positive influence (increased) on the soluble protein content of soybean (Barać & Stanojević, 2005). Whole soybeans soaked in distilled water for one hour required only 4 minutes of microwave treatment at 2450MHZ for inactivation of anti-trypsin factors compared to six minutes that was required for unsoaked soybeans (Yoshida & Kajimoto, 1988). Thus proving microwave treatment to be an effective inactivation method with soaking of the soybeans as an advantageous pre-treatment method.

Radiofrequency treatment is a dielectric heating technology which is less time consuming in comparison with the conventional thermal treatment (Vearasilp, et al., 2005). It is introduced as a novel food processing technique to destroy the trypsin inhibitors and improve the protein quality. In combination with increased temperature, radiofrequency irradiation (27MHz) of soybean samples showed that the TIA levels have been reduced to 7% -12% with an increase in temperatures from 80-120$^o$C and processing time between 90-120 seconds (Vearasilp, et al., 2005). In comparison to MW (2.45GHz, 30 min) and high hydrostatic pressure (600 MPa, $60^0$C, 30 min), RF(27 MHz, 30 min) causes the least nutritional loss (propotion of essential amino acids slightly

increased in dielectric tretaed samples), effectively decreased the antinutritional factors and have relatively less impact on the protein structures (Zhong, Wang, & Zhao, 2015b).

Infrared treatment is another novel processing technique which has an increasing importance in the food industry. This treatment is capable of transferring energy using electromagnetic radiations at lower temperatures. It also improves water adsorption characteristics, reduces cooking time, and also reduces anti-nutritional factors in cowpeas (Kayitesi, Duodu, Minnaar, & de Kock, 2013). It is commercially used in the food industry primarily to reduce the moisture content of grains, legumes, fruits and vegetables (Sakai & Mao, 2006). In a study by Yalcin and Basman (2015) is was showed that infrared treatment (1342 W, 15 min) provided substantial reduction in the trypsin inhibitor activity of pre-soaked whole soybeans (45 min) (Yalcin & Basman, 2015). In this study, laboratory scale infrared equipment was used that consisted of a closed drying chamber, twelve 150 W halogen lamps and two aeration channels.

In food industries, gamma irradiation has been widely used as a disinfectant to get rid of the microorganism. These cause conformational changes, cross linking and aggregation of proteins in food Attempts have been made to use gamma irradiation in the elimination of anti-nutrients of food. In this technique the samples are subjected to a gamma cell which produces these irradiation (Tewari, Kumari, Vinutha, Singh, & Dahuja, 2015). The U.S. Food and Drug Administration has approved the usage of 10 kGy (1 Gy = 1 J $kg^{-1}$) or less gamma irradiation on food for safe human consumption (Tewari, Kumari, Vinutha, Singh, & Dahuja, 2015). Gamma irradiation treatments have also been employed in evaluation of their inhibition capacity in soybeans. Abu-Tarboush, (1998) reported a reduction of 34% in TIA in defatted soybean flour with a dosage of 10 kGy which also improved its digestibility by 4%. This is due to the breakage in trypsin inhibitor structure with the radiation treatment. According to Farag (1998), the inactivation levels increased

with the increase in the dosage of the irradiation. For dosages of 5, 15, 30 and 60 kGy, the loss in the trypsin inhibitory activity was found to be 41%, 56%, 62% and 72.5% respectively. An average of 37% of trypsin inhibitor activity was reduced by 8 kGy gamma irradiation. At legal levels (2 kGy and 4 kGy) the reduction levels were significantly smaller across various cultivars of soybeans used in the study (de Toledo, Canniatti-Brazaca, Arthur, & Piedade, 2007)

Ultrasound is a novel technology in food industry which is still in its infancy. Ultrasound has its applications in many areas including enzyme inactivation, extraction, filtration, drying and homogenization. It reduces processing time when compared to various conventional processing methods(Entezari & Pétrier, 2005). Ultrasound treatment at 20 kHz for about 20 minutes inactivates trypsin inhibitor by 55%. In this study, an ultrasonic homogenizer was used to generate the ultrasonic waves of 20 kHz. The inhibitory activity is directly proportional to the ultrasound amplitudes and sonication duration. It was reported that the inactivation is induced by a reduction in the disulfide bonds and various conformational changes within the protein structure (Huang, Kwok, & Liang, 2008).

High pressure processing (HPP) is another emerging novel processing technique followed in the food industry. The anti-nutritional factors of soymilk are inactivated with a combination of HPP and elevated temperatures as shown in the experiments conducted by (Van Der Ven, et al., 2005) with soymilk samples. High pressure homogenizer was used in the experiment which had a flow rate of 120 L/h. and a high pressure ceramic valve able to support high pressure conditions. A 10% trypsin residual activity was obtained with treatment time of less than two minutes at temperatures between $70^0$C - $90^0$C and pressures between 525 - 750 MPa (Van Der Ven, et al., 2005). A 100% inactivation of trypsin inhibitors was observed by (Linsberger-Martin, Weiglhofer, Thi Phuong, & Berghofer, 2013) at 600 MPa for a holding time of 60 min at $60^0$C in peas.

Shear forces can cause physical deformation of certain protein structures resulting in the change of functional properties of proteins. As trypsin inhibitor's functional properties are dependent on the structural integrity, its disruption can influence the nutritional qualities (Clarke & Wiseman, 2007). Mechanical treatments can be used for inactivation of TI's in various legumes

Extrusion process is one of the major mechanical processing methods used widely in the food industry. Researchers have shown that this process does not change the amino acid composition but has proved to decrease the trypsin inhibitor activity in soy products. Using extrusion methods, soybeans are converted into high quality products without any reduction in the overall protein quality (Clarke & Wiseman, 2007; Romarheim, Aslaksen, Storebakken, Krogdahl, & Skrede, 2005). The most effective process to reduce the inhibitor activity is dry extrusion. The extrusion procedure is based on the temperature impacts appearing during friction of the sample that is squeezed through a cylinder by a specially designed volute. When processed at 150$^{o}$C, the loss of trypsin inhibitor activity was up to 95% of the initial value, whereas the least effective process was wet extrusion treatment(wherein 6-8% of water is added to the ground soybean sample) which retained 40% of the initial TI content (Žilić, 2012).

Instant Controlled Pressure Drop (DIC) is a well-controlled, most recently developed technology involving hydro-thermo-mechanical treatment on soybeans. A 94% inactivation is observed after a minute and 99% is observed after six minutes of DIC treatment ($8\times10^5$ Pa, $50\times10^2$ Pa $170^0$C). The DIC machine has a processing chamber where the samples are treated at high temperature and pressure followed by a reservoir and a valve. The main purpose of this work was to carry out a well- controlled technology for removal of anti-nutrients from food. (Haddad & Allaf, 2007)

Fluidized beds have been successful in reducing the drying time, energy consumption, inactivation of the heat liable inhibitors and also in increasing the nutritional quality of the legume. In fluidized

beds, drying and inactivation of anti-nutritional factors can be achieved in the same process. Osella *et al.*, (1997) investigated the effects of fluidized bed drying on the trypsin inhibitor activities of soybean. At 140$^{o}$C and 10 min, the anti-nutritional factors were completely inactivated from soybean samples in a fluidized bed reactor (Osella, Gordo, González, Tosi, & Ré, 1997)

### 2.3.2 Chemical Treatments

Protease inhibitors of soybean can be reduced by *Nicotinamide adenine dinucleotide phosphate* /thioredoxin system obtained from *Escherichia coli* or plants (consisting of NADP, thioredoxin and NADP-thioredoxin reductase, reduced lipoic acid, dithiothreitol) at 10 mM EDTA at 30$^{0}$C for 1-2 hr in phosphate buffer Kunitz trypsin inhibitor and Bowman-Birk trypsin inhibitors are identified as target proteins of the aforementioned chemical system. The inactivation of the proteins is due to the reduction of NADP/ thioredoxin system by thioredoxin or dithiothreitol (DTT). The relative trypsin activity was 55% and 68% respectively (Jiao, Yee, Kobrehel, & Buchanan, 1992).

L-Ascorbic acid is ubiquitous in plants. Soybeans contain about 40mg/100g of L- Ascorbic acid. The defatted soy flour treated with ascorbic acid and cupric sulfate shows a 90% trypsin inhibitor inactivation at 65$^{0}$C in 1 hr. This is caused mainly due to the pro-oxidant action of ascorbic acid and cupric sulfate directly on trypsin inhibitor (Sessa, Haney, & Nelsen, 1990). Tea polyphenols have proven to have a deactivating effect on soybean trypsin inhibitors. Study conducted by Huang, Kwok, & Liang (2004) shows that polyphenols form a complex with KTI and inactivate them when treated in a water bath at 30$^{0}$C for 30 min.

The heat inactivation of trypsin inhibitors was accelerated by cysteine. The whole soybeans showed a decreased trypsin inhibitory activity up to 90% by the addition of 2.5 mM of cysteine at

pH 9.0, at a temperature of $80^0$C for 10 minutes. The inactivity of the protease inhibitors is due to the disulfide bonds cleavage during the heat treatment. This unfolded protein becomes accessible to cysteine and the inhibitor protein undergoes thiol/disulfide exchange which enhances its denaturation. In the absence of cysteine, this activity was reduced by 30% with the same conditions (Lei, Bassette, & Reeck, 1981).

In addition to a 19% increase in soymilk digestibility, soymilk samples with soybeans pretreated with sodium carbonate showed complete and faster rate of TI inactivation at $98^0$C in 20 min (Wallace, Bannatyne, & Khaleque, 1971). With the effect of sodium hydroxide at $90^0$C for 15 min, the trypsin inhibitors in soybean flour was completely inactivated. The experiment was repeated with different acids and bases at varying concentrations. Sulphuric acid, hydrochloric acid, phosphoric acid, ammonium hydroxide and sodium bicarbonate were also used. Neither Sulphuric acid nor phosphoric acid was as effective as hydrochloric acid in the elimination of trypsin inhibitors. Hydrochloric acid decreased the TI activity at $82^0$C and below, but at all temperatures above $82^0$C, the trypsin inhibitor was stabilized by the addition of acid. Sodium bicarbonate has the least effect, and in comparison with sodium hydroxide the TI was heat liable when cooked at $90^0$C for 15 min with ammonium hydroxide as an additive (Baker & Mustakas, 1973; Wallace, et al., 1971). It was observed that the anti-nutritional activity in soymilk prepared from soybeans soaked in a solution of 0.5% sodium bicarbonate decreased at 550 MPa at $80^0$C with a holding time of 15 min (Guerrero-Beltrán, et al., 2009). A complete inactivation of trypsin inhibitor was observed by (Che Man, Wei, Nelson, & Yamashita, 1991) by soaking whole soybeans in diluted hydrochloric acid at $23^0$C for 8 hours

By using SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) method, it was reported that soybean trypsin inhibitors were inactivated by Ferri sulphas at $70^o$C and pH 7.5

(Baoqing, 2009). Soymeal extracts treated with sulfating agents such as sodium metabisulfite or glutaraldehyde or a combination of both reduces the inhibitory activity of the sample by 95% at $75^0$C in 1 hr ( Sessa, Baker, & Friedrich, 1988).

Table 2 5(a), 2.5(b) and 2.5(c) provides the outline of the effect of different processing treatment on anti-nutrient trypsin inhibitor in soymilk, soy flour and whole soybeans.

Table 2.5 (a): Summary of treatments which have been effective on TIA in whole soybeans

| Treatment | Conditions | Studies |
|---|---|---|
| Autoclaving | $121^0$C, 60 min. | (Osman, Reid, & Weber, 2002) |
| Blanching | $100^0$C, 12 min | (Savage, Wei, Sutherland, & Schmidt, 1995) |
| Boiling | 30 min | (Carvalho & Sgarbieri, 1997) |
| Chemical – Cysteine | $80^0$C, 10 min | (Lei, Bassette, & Reeck, 1981) |
| Chemical- Ferrissulphas | $70^o$C, pH 7.5 | (Baoqing, 2009) |
| Controlled instantaneous pressure drop (DIC) | $8\times10^5$ Pa,$50\times10^2$ Pa, $170^0$C, 1-6 min | (Haddad & Allaf, 2007) |
| Dry extrusion | $150^o$C | (Žilić, 2012) |
| Fluidized bed drying reactor | $140^o$C, 10 min | (Osella, Gordo, González, Tosi, & Ré, 1997) |
| Forced air circulation oven | $150^0$C, 30 min | (Carvalho et al., 2013) |
| Forced Conventional drying | $100^0$C | (Stewart, Raghavan, Orsat, & Golden, 2003) |
| Gamma Radiation | 60 kGy<br>10 kGy<br>8 kGy | (Farag, 1998)<br>(Abu-Tarboush, 1998)<br>(de Toledo, Canniatti-Brazaca, Arthur, & Piedade, 2007) |
| Infrared | 1342 W, 15 min | (Yalcin & Basman, 2015) |
| Micronization | $135^0$C | (Chen, 2015) |
| Microwave | 2.45 GHz, 4 min<br>2.45 GHz, $120^0$C<br>2.45 GHz, 2 min | (Yoshida & Kajimoto, 1988)<br>(Petres et al., 1990)<br>(Barać & Stanojević, 2005) |
| Radio Frequency | 27 MHz, 80-$120^0$C, 1.5 - 2 min | (Vearasilp et al., 2005) |

| | | |
|---|---|---|
| Tea polyphenols | $30^0$C, 30 min | (Huang, Kwok, & Liang, 2004) |
| Ultrasound | 20 kHz, 20 min | (Huang, Kwok, & Liang, 2008) |

Table 2.5 (b): Summary of treatments which have been effective on TIA in soybean flour

| Treatment | Conditions | Studies |
|---|---|---|
| Cooking with Chemical additive- Sodium Hydroxide | $90^0$C, 15 min | (Baker & Mustakas, 1973) |
| Chemical- Ascorbic acid and Cupric sulfate | $65^0$C,1 hr | (Sessa, Haney, & Nelsen, 1990) |
| Hot Oven | $200^0$C, 20 min | (Andrade, Mandarino, Kurozawa, & Ida, 2016) |
| Hot air drying | $100^0$C for 2 hr | (Agrahar-Murugkar & Jha, 2010) |

Table 2.5 (c): Summary of treatments which have been effective on TIA in soymilk

| Treatment | Conditions | Studies |
|---|---|---|
| Cooking with Chemical additive- Sodium carbonate | $98^0$C, 20 min | (Wallace, Bannatyne, & Khaleque, 1971) |
| High pressure Processing | $70^0$C-$90^0$C, 525- 750 MPa, 2 min | (Van Der Ven, Matser, & Van Den Berg, 2005) |
| Ultra-High Temperature (UHT) | $125^0$C, 5min | (Kwok, Liang, & Niranjan, 2002) |
| Ohmic Heating | 220V, 50Hz, 3 min | (Lu et al., 2015) |
| Chemical- 0.5% sodium bicarbonate | $80^0$C,550 MPa, 15 min | (Guerrero-Beltrán, Estrada-Girón, Swanson, & Barbosa-Cánovas, 2009) |
| Adsorption- Allyl glycidyl ether-ethylene glycol dimethacrylate copolymer with 25 % crosslink density | 100 rpm, 1 hr | (Jasti, Lavanya, & Fadnavis, 2015) |
| Grinding and Blanching | $100^0$C, 15 min | (Murugkar, 2015) |

## 2.4 FUTURE – MOLECULAR MODELING

Scientists have always believed that the functional properties of a protein are always connected to its structure. Thus, when proteins are processed the external stress leads to conformational changes in the molecular structure directly affecting the functional properties as discussed throughout this review (Davis & Williams, 1998; Singh, et al., 2015). Techniques like Fourier Transformation Infrared Spectroscopy (FTIR), Nuclear Magnetic Resonance Imaging (NMR), X-ray diffraction and Circular Dichroism can be employed to analyze the structure of these proteins, but these techniques are expensive. Moreover, only the initial and final structures can be analysed using the above mentioned techniques which limits the ability to understand the exact mechanism involved in secondary structure deviations of the protein. To overcome these short comings, molecular modeling techniques can be applied to primarily visualize the structural changes within the protein or enzymes during processing (Budi, Legge, Treutlein, & Yarovsky, 2005; Vanga, Singh, & Raghavan, 2015a). This technique is widely used to investigate the dynamic and structural properties of biomolecules and nanoparticles in the field of pharmacology, molecular biology, and mechanical engineering to test and develop new products (Lorenzo & Caffarena, 2005; Vanga, Singh, et al., 2015b; Vriend, 1990).

Astrakas *et al.* evaluated the effects of external electric fields on the conformation of Chignolin β-hairpin protein. They showed that the external electric fields can disrupt the stability of Chignolin protein. Under the external stress, the protein molecule first tried to orient in the direction of the field due to the changes in the dipole moment. Further, they concluded that the hydrogen bonds are disrupted and the terminal residues of the protein have separated under continuous external stress (Astrakas, Gousias, & Tzaphlidou, 2011).

Singh *et al.*, also used this technique to study the surface properties of Soybean Hydrophobic Protein (SHP) (Singh, Orsat, & Raghavan, 2013). In this study, an electric field strength between 0.002 V/nm – 3 V/nm has been applied on the protein and the changes in the secondary structure have been evaluated. They concluded that when a static electric field of lower orders are applied, the protein biomolecule under stress would reorient itself into most stable state. But, when a higher voltage is applied, the molecule is broken down and all the secondary structures are lost as a result of the static electric field. These changes in the secondary structure are shown in terms of Root Mean Square Deviation (RMSD), Radius of Gyration (Rg) and Solvent Accessible Surface Area (SASA). These simulations can be used to generate useful data in understanding how various protein biomolecules reorient themselves under external stresses.

Recently, Vanga *et al.,* have applied similar molecular modeling techniques to evaluate the secondary structure changes in the peanut allergen Ara h 6 (Vanga, Singh, et al., 2015a). They evaluated the changes in the secondary structure due to thermal and electric fields and their combinations. Budi *et al.,* evaluated the effect of electric field on the enzyme insulin chain-B's structural conformation. They further showed that oscillating electric fields resulted in higher disruptions of secondary structures which could lead to reduced access to the active sites on the insulin enzyme (Budi, et al., 2005; Budi, Legge, Treutlein, & Yarovsky, 2007). Similar techniques can also be applied to study the anti-nutritional factors like trypsin inhibitors to evaluate the effect of processing on the soybean protein structure

## 2.5 CONCLUSION

This review discusses the effects of processing on phytochemical properties of the trypsin inhibitor which is mainly present in soybeans. Digestibility of soy protein is primarily hindered due to the presence of soybean trypsin inhibitors and their inactivation is necessary for improving the nutrient value. The review highlights the various novel and conventional processing methods adopted to improve and increase the digestibility and the nutritional value of soy protein. Apart from increasing the nutritional value, the food researchers should also make an effort to encourage the public to consume soy foods. For understanding the structural mechanism involved, molecular modelling techniques can be used. These techniques will help us understanding the structural changes in the protein and the enzyme during various processing.

## CONNECTING TEXT

In the review, we have seen the various novel and conventional processing methods adopted to improve the nutritional quality of soy products. Due to the absence of upper safe limits for TI's in food products, their elimination is a major concern. The next part of the thesis deals with the application of microwave processing of raw soymilk for elimination of anti-nutritional factors (TI's). The processing time of conventional methods are prolonged which damage the protein quality. Hence, in order to increase the *in-vitro* protein digestibility and reduce the trypsin inhibitor activity of the protein, a potential alternative processing method is considered that is less time consuming. The results are compared to the conventional thermal treatment and experimental conditions are optimized using Response Surface Methodology with central composite design.

## CHAPTER III

## Comparison of Conventional and Microwave Treatments of Soymilk for Inactivation of Trypsin Inhibitors and *In-vitro* Protein Digestibility

### 3.1 Abstract

Soymilk is an excellent source of protein derived from a plant source and also is lower in calories and cholesterol when compared to cow's milk. In spite of the beneficial factors, soymilk is considered as one of the most controversial foods in the world. It contains serine protease inhibitors which lower its nutritional value and digestibility. Processing techniques for the elimination of trypsin inhibitors and lipoxygenase, that have shorter processing time and lower production costs, are required for the large scale manufacturing of soymilk. In this study, the suitable conditions of time and temperature for microwave processing were optimized to obtain soymilk with maximum digestibility with inactivation of trypsin inhibitors, in comparison to the conventional thermal treatment. The microwave processing at a frequency of 2.45 GHz and at temperatures $70^0$C, $85^0$C and $100^0$C for 2, 5 and 8 min were investigated, and compared to conventional thermal treatments at the same temperatures for 10, 20 and 30 min. Response surface methodology was used to design the experiments and the processing conditions were optimized.

**KEYWORDS:** Soymilk, Microwave Processing, Thermal Processing, Trypsin inhibitors, Response Surface Methodology.

## 3.2 Introduction

Soymilk is a high protein liquid with considerable amounts of carbohydrates, fats, essential vitamins and minerals, and is generally produced by grinding soaked soybeans in excess amount of water, which is then filtered to separate out the milk from solids and fibre. It is a stable oil in water emulsion, where the continuous phase is formed by the dispersed soybean protein. Soymilk is composed of 94% water, 3% protein, 1.5% fat and 1.5% of carbohydrates. It also contains 7.36 and 0.33 mg/100 mL of riboflavin and thiamin respectively, a composition similar to cow's milk but with little saturated fat and no cholesterol (Buzzell, 1987; Jasti et al., 2015; Lakshmanan, De Lamballerie, & Jung, 2006). The nutritional profile of soymilk is summarized in Table 3.1.

**Table 3.1**: Nutritional profile of soymilk (unfortified)

| NUTRIENTS | Value per 100 g |
|---|---|
| Water | 85.61 |
| Protein | 2.26 |
| Dietary Fiber | 0.4 |
| Calcium | 0.025 |
| Carbohydrates | 9.95 |
| Sugars | 7.86 |
| Potassium | 0.143 |
| Cholesterol | 0 |
| Trans Fatty acids | 0 |

(Source: Report: 16166, USDA Database)

In recent years the consumption of soymilk has been increasing especially among consumers who are lactose intolerant, vegetarians, vegans and/ or seeking healthy diets. It is also considered safe for children with galactosemia (Friedman & Brandon, 2001), as galactose is absent in soymilk. In

developing countries, soymilk is used as a low cost substitute to cow's milk in many food preparations (Cruz et al., 2007; Reilly, Lanou, Barnard, Seidl, & Green, 2006). This increase in consumption of soymilk can also be attributed to the presence of high quality protein and low fat content (Lakshmanan et al., 2006). In 1999, the U.S Food and Drug Administration approved the health claim for soy protein, which states that its consumption may reduce the risk of heart diseases by lowering the levels of low density lipoproteins, adding to its acceptance by wide variety of consumers (Min, Yu, & Martin, 2005). Several researchers have also associated the consumption of soy products to the reduced risks of coronary heart diseases, atherosclerosis, type 2 diabetes, colorectal cancer, breast cancer and prostate cancer (Anderson & Wolf, 1995; Hwang, Kim, Jee, Kim, & Nam, 2009; Kerwin, 2004).

In spite of all the aforementioned beneficial factors, the nutritional value of soy milk is reduced by the presence of a variety of antinutritional factors such as Kunitz trypsin inhibitors (KTI), Bowman- Birk inhibitors (BBI) and lipoxygenase (LOX). Soybean contains the highest amount of protease inhibitors that accounts for two to six percent of the whole soybean protein (Vinh & Dworschak, 1986). These proteases (KTI and BBI) inhibit the enzymatic activity of trypsin and chymotrypsin, the primary digestive enzymes responsible for reducing the proteins into dipeptides and tripeptides. The KTI has a molecular weight of 20 kDa with two disulfide bridges and exhibits specificity to inhibit trypsin. BBI has a molecular weight of 8 kDa with seven disulfide bonds and exhibits specificity to inhibit chymotrypsin and trypsin (Liener, 1994). Rouhana *et al.*, reported 60% of soymilk trypsin inhibition activity is from KTI (Rouhana et al., 1996). High levels of active KTI have been shown to reduce protein digestibility and cause pancreatic carcinogenesis upon consumption (Xiao et al., 2012). In animals, protease inhibitors have been associated with growth suppression and pancreatic hypertrophy, emphasizing the need for identification and development

of effective techniques to reduce their presence in soy products (Friedman, Brandon, Bates, & Hymowitz, 1991; Grant, 1988; Van Der Ven et al., 2005). Soybean trypsin inhibitors are heat stable and require a long processing time. According to Yuan *et al.,* TIA decreased to 13% of the original raw soymilk concentration by the traditional processing method (heating at $100^0$C for 20 min) (Yuan, Chang, Liu, & Xu, 2008). But the long processing time may affect the other nutritional properties of soy products and hence has to be avoided. At the same time, 100% inactivation of trypsin inhibitors through overheating destroys lysine, tryptophan and cysteine amino acids in soymilk. It also leads to the browning reaction and other deteriorative reactions. The flavour, colour, and vitamin content are also affected depending on the type of heat treatment used (Adams, 1991; Agrahar-Murugkar & Jha, 2010). Hence, processing plays an essential role in the sensory appeal and nutritive value of soybean and soy products including soymilk. The various factors to be considered for a good quality soymilk through processing are yield, nutritional quality, antinutritional profile, colour attributes, particle size, texture profile and organoleptic quality (Sancho et al., 1999).

Moreover, there are still questions remaining concerning the ideal processing conditions to produce commercially sterile soymilk with minimum nutrient degradation. We require manufacturing techniques that have shorter processing time, are energy efficient (environment friendly), have lower production costs and maintain the quality of soymilk (Chen, Xu, Zhang, Kong, & Hua, 2014). Autoclaving, batch boiling and steam injection (Murugkar, 2015; Wallace et al., 1971), ultra-high temperature (UHT) (Kwok, Liang, & Niranjan., 2002), high temperature and pressure combination (Guerrero-Beltrán et al., 2009), Ohmic heating (Lu et al., 2015), and high pressure processing (HPP) (Van Der Ven et al., 2005) are processing methods that have been explored for the inactivation of trypsin inhibitors in soymilk.

Industrial scale dielectric heat treatment technology at 42 MHz (Radio frequency) and 2450 MHz (Microwave) were found to be effective against trypsin inhibitors in soybean and in addition, these methods also improved the overall quality of the soy proteins. The processing time required to reach safe levels of trypsin inhibitor inactivation is less in microwave treatment when compared to conventional methods for soybeans (Kala & Mohan, 2012). Studies by Barac and his team showed that the trypsin inhibitor levels were reduced to 13% of the initial value in soybean during microwave roasting at 2.45 GHz for 2 minutes (Barać & Stanojević, 2005). In a study conducted by Yoshida *et al.,* the inactivation of the antinutritional factors to safe limits of soaked soybean at 2.45 GHz requires only 4 minutes (Yoshida & Kajimoto, 1988). In comparison, conventional batch boiling process takes 15 minutes at $100^0$C to inactivate the levels of trypsin inhibitor by 20% (Rouhana et al., 1996). To the best of our knowledge, no studies have been done on the inactivation of soybean trypsin inhibitor in soymilk using microwave processing, in spite of the various advantages of using this dielectric processing techniques in food processing. This can be regarded as a potential alternative to the existing conventional processing methods in food industry for inactivation of antinutritional factors.

This study reported the effects of microwave processing in reduction of trypsin inhibitor activity in comparison to conventional thermal processing of soymilk. The *in vitro* protein digestibility (IVPD) studies were also performed to assess the effects of microwave processing at different time and temperature conditions on the digestibility of soymilk. Optimization of these processing techniques were performed using response surface analysis method.

### 3.3 Materials and Methods

Soybeans (*Glycine max*) was procured from Goliath, QC, Canada. Initial moisture content was found as 10.1% on wet basis. The moisture content was determined by following the AOAC

official method for moisture content analysis in soybean flour using hot air oven. Soybean flour (5 g) was dried in an oven at $130^0$C $\pm 3^0$C for 2 hours, after which the weight became constant (Official methods of Analysis of AOAC, 2006)(International, Horwitz, & Latimer, 2006). Fresh soymilk was prepared from soybeans before performing thermal and microwave processing.

### 3.3.1 Soymilk Preparation

Soybeans were washed, cleaned and soaked in distilled water in the ratio 1:10 (bean: water) for 18 hours at room temperature ($25^0$C) for complete hydration. The soymilk was prepared by wet grinding of the hydrated soybeans along with water for 3 mins at high speed in a stainless steel blender. The slurry was filtered through a double layer of cheesecloth to separate out the solids from soymilk. Raw soymilk obtained had a pH of 6.5 (Min et al., 2005).

### 3.3.2 Solvents and Reagents

All reagents and solvent used were of HPLC grade and were purchased from Fisher Scientific (Ottawa, ON, Canada). The enzymes used for IVPD % determination and trypsin inhibitor assay were purchased from Sigma Aldrich (Oakville, ON, Canada),

### 3.3.3 Conventional thermal treatment

For conventional thermal treatment, 30 mL of soymilk was placed in a water bath which was pre-set and maintained at the processing temperatures of $70^0$C, $85^0$C and $100^0$C. The samples were treated for 10, 20 and 30 minutes in the water bath. All the experiments were conducted in triplicates. After cooling at room temperature, the samples were collected and stored overnight at $-40^0$C. The processed soymilk samples were later freeze dried in a laboratory freeze-dryer (Christ Gamma 1-16 LSC Freeze dryer) and stored in opaque air-tight containers at $-20^0$C until further analysis was conducted.

### 3.3.4 Microwave processing

The microwave processing was conducted using the MiniWAVE digestion system (SCP Science, Canada) that operates at a frequency of 2.45 GHz. The soymilk samples (50 mL) were heated in cylindrical quartz reactor vessels. The experiments were conducted at processing temperatures of $70^0$C, $85^0$C and $100^0$C for 2, 5 and 8 minutes. The sample temperature was monitored using IR sensors located on the sidewalls and displayed in real time on the controller screen during the treatment. After the treatment, the reactor vessels were cooled to room temperature gradually by the cooling unit of the microwave system. The samples were stored until further analysis in the same manner as that of conventionally treated samples.

### 3.3.5 Chemical Analysis

#### 3.3.5.1 Trypsin Inhibitor Assay

In this study, the total trypsin inhibitor activity was assessed using the procedure followed by Hamerstrand *et al.* with some modifications (Embaby, 2010; Hamerstrand, Black, & Glover, 1981). Freeze dried soy milk (0.5 g) was extracted with 50 mL of 0.01 M NaOH for 3 hours, with constant stirring at room temperature. The suspension was then allowed to stand for two hours at $4^0$C. The supernatant from each sample was collected and diluted, such that two microliters of the extract could produce 40-60% trypsin inhibitor activity.

Trypsin (type 1X from bovine pancreas, Sigma Chemical Co) was used as a standard. Diluted soymilk supernatant (1 mL) was pipetted into test tubes in triplicates containing two microliters of trypsin solution (20 mg in 0.001 M HCl). The control sample (blank) consisted of diluted sample extract and distilled water. The tubes were preheated at $37^0$C for 10 min and then, five microliters of benzyl- DL-arginine- para- nitroanilide (BAPNA), pre-warmed to $37^0$C, and was added to each

of the tubes and vortexed. After incubating this mixture at $37^0$C for 10 min, the reaction was stopped by adding 1 mL of aqueous acetic acid (30%, mL/mL). The samples were centrifuged at 3000 g for 10 min. The absorbance of the clear supernatant was measured at 410 nm using spectrophotometer (Kakade, Rackis, McGhee, & Puski, 1974a). Trypsin inhibitor activity (TIA) is calculated using Equation (1) in terms of pure trypsin /g sample as weighed (mg/g).

$$TIA = (2.632 \times D \times A_I) / S \quad (1)$$

Where, D is the dilution factor (factor by which the original soymilk sample was diluted so as to obtain an inhibition between 40%-60% by 1 mL of the diluted extract), S is the sample weight and $A_I$ is the change in absorbance due to trypsin inhibitor/ mL diluted sample extracted.

**3.3.5.2 In-vitro Protein Digestibility (Multi Enzyme Method)**

The *in-vitro* protein digestibility (IVPD) of soybean protein was evaluated using the multi-enzyme method. The working protein suspension was prepared by dissolving samples to yield 312.5 mg of protein in 50 mL of distilled water, whose pH was adjusted to 8 using 0.1 N NaOH and 0.1 N HCL. A multi-enzyme mixture was prepared, containing 1.6 mg/ml trypsin, 3.6 mg/ml chymotrypsin, and 1.3 mg/ml peptidase and its pH was adjusted to 8. The mixture was placed in an ice-bath and continuously stirred (Astwood et al., 1996; Bodwell, Satterlee, & Hackler, 1980; Hsu, Vavak, Satterlee, & Miller, 1977; Vanga, Singh, Kalkan, et al., 2015b). Five microliters of the multi-enzyme solution was added to the samples, which were maintained at $37^0$C in a water bath for the digestion with continuous stirring. The pH was measured after 10 minutes of the digestion and the percent IVPD was calculated using Equation 2 (Hsu et al., 1977).

$$IVPD\ \% = 210.46 - (18.10 \times pH_{10min}) \quad (2)$$

### 3.3.6 Experiment design and analysis of the results

In this study, the response surface methodology was employed for the design of experiments, fitting of mathematical model and optimization of processing conditions for soymilk samples. The central composite design (CCD) with uniform precision was applied for two independent factors, namely temperature ($X_1$) and time ($X_2$), each at three levels (-1, 0, +1) as shown in Table 3.2. The experiment design consisted of a total of 14 combinations with six central, four factorial and four axial point combinations as shown in Table 3.3.The responses: trypsin inhibitor activity and in-vitro protein digestibility were recorded. JMP software version 11 (SAS Institute Inc., Cary, NC, USA) was used for the experimental design and analysis. The functional relationship between the factors ($X_i$,$X_j$,$X_k$....) and responses ($Y$) were unknown, hence a regression model (Equation 3) was used to analyse the actual response surfaces (Myers, Montgomery, & Anderson-Cook, 2009).

$$Y = \beta_0 + \sum_{i=1}^{i=n} \beta_i X_i + \sum_{j=1}^{j=n} \beta_j X_j + \sum_{i=1}^{i=n} \beta_{ii} X_i^2 + \sum_{j=1}^{j=n} \beta_{jj} X_j^2 + \sum_{i=1}^{i=n} \sum_{j=1}^{j=n} \beta_{ij} X_i X_j \ldots\ldots\ldots \quad (3)$$

Where, $\beta_0$ is the intercept, $\beta_i$ is the linear coefficient for the variable $i$, $\beta_j$ is the linear coefficient for the variable $j$, $\beta_{ii}$ is the quadratic coefficient for the variable $i$, $\beta_{jj}$ is the quadratic coefficient for the variable $j$ and $\beta_{ij}$ is the second order interaction coefficient of variables $i$ and $j$, respectively.

**Table 3. 2:** Central composite design for processing of soymilk with independent variables and their coded and actual values

| Process parameters | Units | Coded levels | | |
|---|---|---|---|---|
| | | -1 | 0 | +1 |
| Temperature | $^0$C | 70 | 85 | 100 |
| Time (Microwave processing) | Min | 02 | 05 | 08 |
| Time (Conventional water bath) | Min | 10 | 20 | 30 |

**Table 3.3:** Central composite design showing different combinations of temperature and time for processing of soymilk

| Experimental run | Temperature ($^0$C) | Time (min) Microwave Processing | Time (min) Conventional Processing |
|---|---|---|---|
| 1 | 70 | 2 | 10 |
| 2 | 70 | 5 | 20 |
| 3 | 70 | 8 | 30 |
| 4 | 85 | 2 | 10 |
| 5 | 85 | 8 | 30 |
| 6 | 100 | 2 | 10 |
| 7 | 100 | 5 | 20 |
| 8 | 100 | 8 | 30 |
| 9-14 | 85 | 5 | 20 |

The experiment design was prepared taking temperature in $^0$C and time in min. Separate CCD was prepared for both conventional thermal processing and microwave processing methods. The F value and its significance ($p \leq 0.05$), Lack of Fit (LOF), coefficient of determination ($R^2$) were assessed and the ANOVA with the corresponding significant terms are reported in Tables 3.4, 3.5, 3.6, 3.7, 3.8 and 3.9. The significant differences among the treatments were also detected using Duncan multiple-range test using the probability level 0.05 (Su & Chang, 2002).

## 3.4 Results and Discussions

### 3.4.1 Optimization of conditions for the maximum *in-vitro* protein digestibility (IVPD) during microwave and conventional processing

Legume proteins are known to have a lower digestibility, which is attributed to the presence of antinutritional factors. On an average, IVPD of microwave processed and conventionally treated soymilk samples in this experiment was 85% and 88% respectively as shown in Table 3.4. The nutritional quality of a soybean protein cannot be determined by its amino acid composition alone, but its digestibility in the small intestine that determine the amino acid bioavailability should also be taken into account. Our investigations on the microwave processing of soymilk indicated that

both the independent factors; temperature (temp) ($p < 0.0001$) and time (t) ($p < 0.0237$) significantly influenced the IVPD of soy proteins.

**Table 3.4:** Summarized statistics for in-vitro protein digestibility of the processed soymilk

| | Microwave processing | Conventional processing |
|---|---|---|
| Range (% digestibility) | 82-89 | 84-92 |
| Average | 85.2183 | 88 |
| Standard deviation | ±1.4586 | ±2.018 |

**Table 3.5:** ANOVA for effect of time (t) and temperature (temp) on in-vitro protein digestibility of microwave processed soymilk

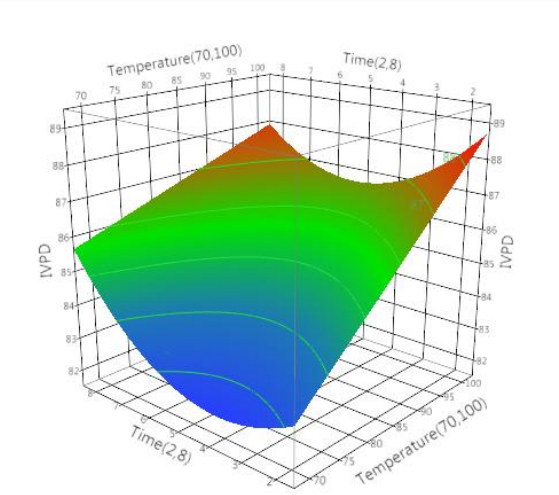
| Source | DF | Sum of squares | Mean square | F ratio | p-value |
|---|---|---|---|---|---|
| Model | 4 | 74.5361 | 18.6340 | 46.5061 | <0.0001 |
| Temperature (temp) | 1 | 51.6754 | | 128.9695 | <0.0001 |
| Time (t) | 1 | 2.2296 | | 5.5645 | 0.0237 |
| Temp × t | 1 | 4.5892 | | 11.4537 | 0.0017 |
| $t^2$ | 1 | 16.0418 | | 40.0366 | <0.0001 |
| Lack of fit | 4 | 3.4025 | 0.8506 | 2.4575 | 0.0648 |
| Error | 37 | 14.8251 | 0.4007 | | |
| C. Total | 41 | 89.3613 | | | |
| Pure Error | 33 | 11.4225 | 0.3461 | | |
| Total Error | 37 | 14.8251 | | | |

From Table 3.5 it can also be observed that the quadratic effect of time ($t^2$) ($p < 0.0017$) and the interaction effect of temperature (temp) and time (t) ($p \leq 0.0001$) too significantly influenced the IVPD.

The reduced model containing the significant model terms for $IVPD_{Microwave}$ can be described in terms of significant coded factors as shown in Equation 4. It can be seen that the model F-ratio of 46.51 implies that the model is significant and there is only a 0.01% chance that F-ratio this large could occur due to noise. The lack of fit F-ratio of 2.46 and its probability of *p*-value of 0.648 also implies that an insignificant lack of fit this large would not have occurred due to noise.

$$IVPD_{microwave} = 84.68 + 1.69Temp + 0.35t + 1.25t^2 - 0.62Temp * t$$
$$R^2 = 0.83 \quad (4)$$

**Figure 3.1** Effect of time (min) and temperature (°C) of microwave processing on *in-vitro* protein digestibility (IVPD) of soymilk




The developed regression model was further used to illustrate the relationship between the independent and dependent factors via three-dimensional response surfaces. The response surface graphs for microwave processing of soymilk showed that as the microwave processing time was increased at any processing temperature, a slight decrease in IVPD was observed till the time reached approximately 5 min and later the IVPD increased as represented in Figure 3.1.

This observation led to a conclusion that can be related to the changes in the conformation of proteins under the influence of oscillating electric field of microwave (2.4 GHz). When the sample was subjected to microwave processing, a change in the conformation of soymilk proteins might have reduced its susceptibility to the digestive enzymes and when the processing time increased,

the proteins were denatured and the digestion was proceeded as desired. This conclusion is based on previous observations made by the researchers though molecular modeling studies conducted to evaluate the effect of oscillating and static electric fields on various food proteins including peanut and soybean hydrophobic proteins (Singh, Orsat, et al., 2013; Vanga, Singh, & Raghavan, 2015a). Similar studies were conducted to understand the structure and digestibility of proteins in other legumes such as dry beans (*Phaseolus vulgaris*) and green peas (*Pisum sativum*) (Deshpande & Damodaran, 1989), sorghum (*Sorghum bicolor*) and maize (*Zea mays*) (Duodu et al., 2001; Duodu, Taylor, Belton, & Hamaker, 2003).

**Table 3.6:** ANOVA for effect of time (t) and temperature (temp) for in-vitro protein digestibility for conventional processing

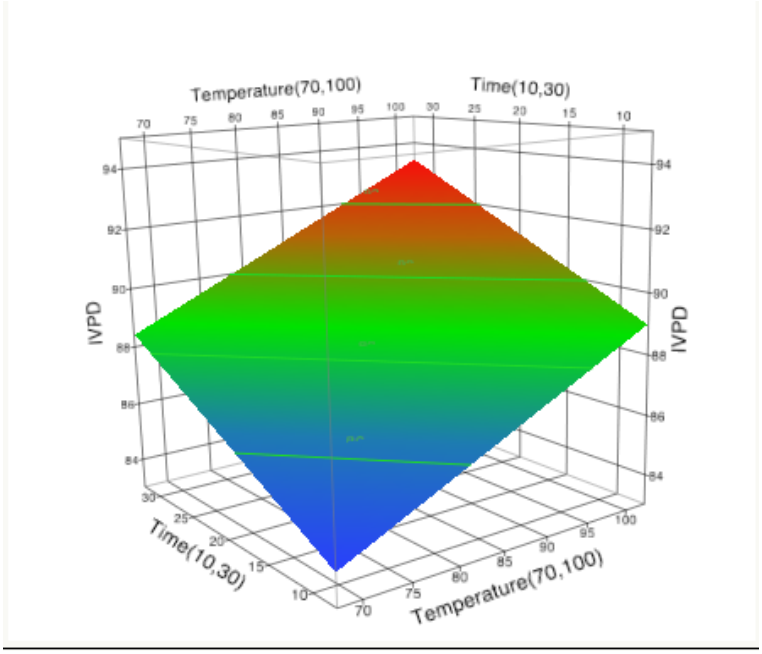
| Source | DF | Sum of squares | Mean square | F ratio | p-value |
|---|---|---|---|---|---|
| Model | 2 | 137.5613 | 68.7807 | 79.9635 | <0.0001 |
| Temperature (temp) | 1 | 81.4675 | | 94.6810 | <0.0001 |
| Time (t) | 1 | 56.0940 | | 65.1919 | <0.0001 |
| Lack of fit | 6 | 4.2183 | 0.7030 | 0.7908 | 0.5836 |
| Error | 39 | 33.5572 | 0.8604 | | |
| C. Total | 41 | 171.1185 | | | |
| Pure Error | 33 | 29.3388 | 0.8891 | | |
| Total Error | 39 | 33.5572 | | | |

Similarly, for conventional thermal processing of soymilk, it was observed that only the linear effect of temperature (temp) and time (t) were significant ($p < 0.0001$) and positive on the IVPD of soy proteins, and the interactions and quadratic effects were not significant ($p>0.05$) (Table 3.6). The response surface graph for conventional processing of soymilk is shown in Figure 3.2.

Equation 5 shows the reduced model for the conventional treatment ($IVPD_{Conventional}$) of soymilk in terms significant model terms

$$IVPD_{Conventional} = 88.73 + 2.13Temp + 1.77t \qquad R^2 = 0.80 \tag{5}$$

**Figure 3.2** Effect of time (min) and temperature ($^0$C) of conventional thermal treatment on *in-vitro* protein digestibility (IVPD) of soymilk




Several researchers have suggested that the treatment temperature is the key determinant of food protein digestibility (Bax et al., 2012; Zhang et al., 2013). Similar linear relationships between treatment time and temperature were observed by Wallace *et al* in their study on effect of different heat processing conditions on the trypsin inhibitor activity and the IVPD of various soymilk preparations. They reported that the digestibility of proteins increased with an increase of temperature in the heat treatment and coincided with a decrease in the trypsin inhibitor activity

(Wallace et al., 1971*)* . Our study also showed similar results. It was observed that maximum digestibility of soymilk proteins occurred at $100^0$C for 30 mins of conventional processing. Other studies establishing the relationship to increase in digestibility due to decrease in antinutritional factors were seen in rice (Sagum & Arcot, 2000), cowpea (Laurena, Garcia, Mae, & Mendoza, 1987), chickpeas (Alajaji & El-Adawy, 2006), moth beans (Negi, Boora, & Khetarpaul, 2001), and common beans (Marconi, Ruggeri, Cappelloni, Leonardi, & Carnovale, 2000).

### 3.4.2 Optimization of conditions for reduced trypsin inhibitor activity (TIA) during microwave and conventional processing

Trypsin inhibitor activity governs the nutritional value of soymilk proteins (Hackler, Buren, Steinkraus, Rawi, & Hand, 1965; Lei et al., 1981). The average values for TIA of soymilk after microwave and conventional processing is presented in Table 3.7. It has been reported by several researchers that the overheating for complete removal of TIA reduces the overall nutritive value of soybeans (Skrede & Krogdahl, 1985). Hence, a novel precisely *controlled* thermal process is required for preparation of soymilk with maximum nutritive value.

In this study, analysis of the effects of microwave and conventional processing of soymilk on TIA revealed that both temperature and time play a significant ($p<0.05$) role in it. Table 3.8 presents the ANOVA of the effect of temperature and time on TIA of soymilk after microwave processing. From the table it can be observed that temperature (temp) and time (t) significantly influenced the TIA ($p < 0.0001$ and $p < 0.0009$, respectively) as did the quadratic effect of temperature ($p \leq 0.0001$), whereas neither the quadratic effect of time nor its interaction with temperature had any effect ($p > 0.05$) on the TIA of the processed soymilk**.**

**Table 3.7:** Summarized statistics for trypsin inhibition activity of soymilk processing using process parameters according to central composite design

| | Microwave processing | Conventional processing |
|---|---|---|
| Range (% inhibition) | 3-18.8 | 1-9 |
| Average | 11.6 | 3.1 |
| Standard deviation | ±4.0333 | ±2.002 |

**Table 3.8:** ANOVA for effect of time (t) and temperature (temp) on trypsin inhibitor activity for microwave processing

| Source | DF | Sum of squares | Mean square | F ratio | p-value |
|---|---|---|---|---|---|
| Model | 3 | 6.2115 | 2.0705 | 126.6718 | <0.0001 |
| Temperature (temp) | 1 | 5.6224 | | 343.9744 | <0.0001 |
| Time (t) | 1 | 0.2112 | | 12.9241 | 0.0009 |
| $Temp^2$ | 1 | 0.3778 | | 23.1169 | <0.0001 |
| Lack of fit | 5 | 0.0814 | 0.0163 | 0.9956 | 0.4356 |
| Error | 38 | 0.5871 | 0.0163 | | |
| C. Total | 41 | 6.8326 | | | |
| Pure Error | 33 | 0.5397 | 0.0163 | | |
| Total Error | 38 | 0.6211 | | | |

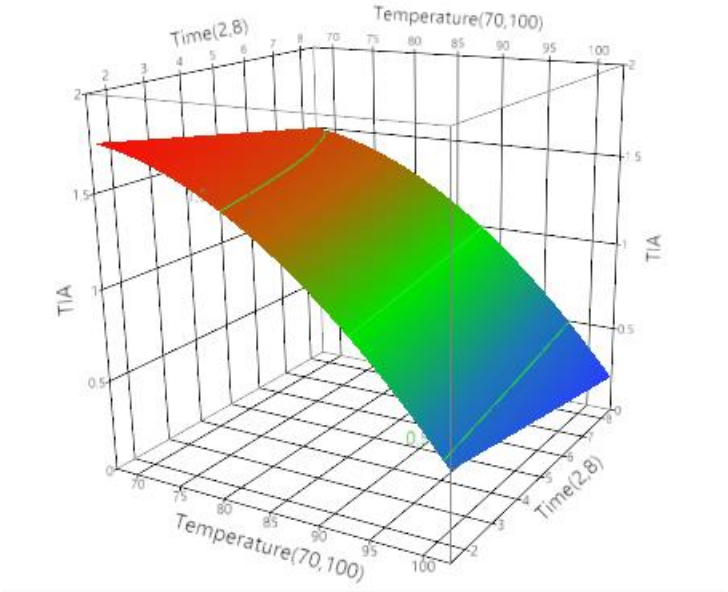
**Table 3.9**: ANOVA for effect of time (t) and temperature (temp) for trypsin inhibitor activity for conventional processing

| Source | DF | Sum of squares | Mean square | F ratio | p-value |
|---|---|---|---|---|---|
| Model | 5 | 1.3021 | 0.2604 | 24.5188 | <0.0001 |
| Temperature (temp) | 1 | 0.5408 | | 50.9176 | <0.0001 |
| Time (t) | 1 | 0.3472 | | 32.6918 | <0.0001 |
| $Temp^2$ | 1 | 0.1180 | | 11.1108 | 0.0020 |
| Temp × t | 1 | 0.0835 | | 7.8624 | 0.0081 |
| $t^2$ | 1 | 0.0892 | | 8.4001 | 0.0064 |
| Lack of fit | 3 | 0.2551 | 0.0850 | 22.0541 | <0.0001 |
| Error | 36 | 0.3823 | 0.0106 | | |
| C. Total | 41 | 1.6844 | | | |
| Pure Error | 33 | 0.1272 | 0.0040 | | |
| Total Error | 36 | 0.3823 | | | |

The reduced model containing the significant model terms developed for $TIA_{Microwave}$ can be described in terms of coded factors as shown in Equation 6.

$$TIA_{Microwave} = 1.25 - 0.56Temp - 0.11t - 0.19Temp^2$$
$$R^2 = 0.90 \quad (6)$$

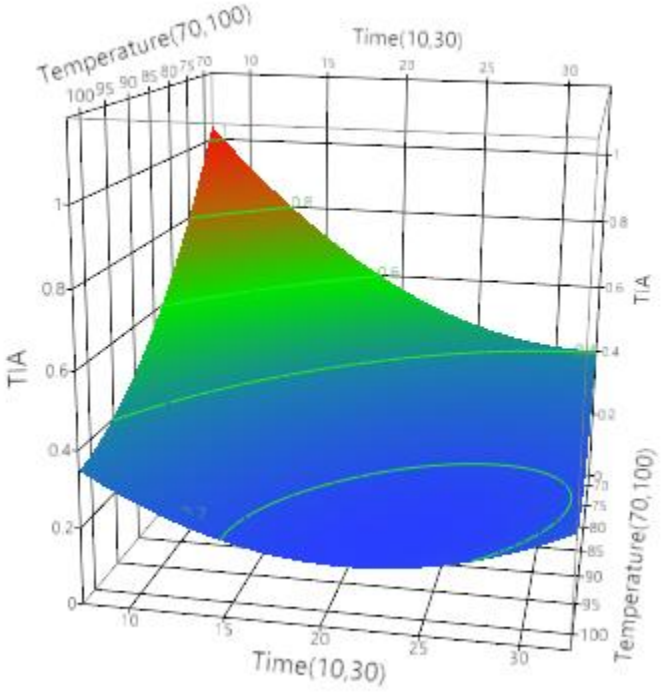
**Figure 3.3** Effect of time (min) and temperature ($^0$C) of microwave processing on trypsin inhibitor activity (TIA) of soymilk




The response surface analysis further suggested that trypsin inhibitor activity decreases with increase in temperature and time (Figure 3.3), but the significant temperature square ($Temp^2$) term suggested that the temperature of the treatment significantly influence the trypsin inhibitor activity. Studies by Rajko *et al*, have shown that the inactivation of trypsin inhibitors requires more absorbed heat energy from longer processing time (Rajkó, Szabó, Vidal-Valverde, & Kovács, 1997). Similar experimental results were obtained by Alajaji and El-Adawy, during the microwave

oven cooking of chickpea at 2.45 GHz for 15 min (Alajaji & El-Adawy, 2006). According to the studies by Oliveria and Haghighi, reduction in TIA was more pronounced in samples treated at higher temperatures because soybean TIA loses irreversibly at the temperature range of 80–110$^0$C (Oliveira & Haghighi, 1998). Also, studies by Esaka *et al.*, reported that the trypsin inhibitor activity was not detectable after microwave heating at 120$^0$C for 5 min in the case of winged bean seeds (Esaka et al., 1987).

**Figure 3.4** Effect of time (min) and temperature ($^0$C) of conventional thermal treatment on trypsin inhibitor activity (TIA) of soymilk



Similarly analysis of the effect of conventional processing of soymilk on TIA shown that all the factors including their respective quadratic and interaction effects significantly affected it ($p<0.05$). Equation 7 presents the reduced model containing the significant model terms obtained for the effect of conventional processing of soymilk on TIA ($TIA_{Conventional}$).

$$TIA_{Conventional} = 0.23 - 0.17Temp - 0.14t + 0.099Temp^2 + 0.10t^2 + 0.099Temp*t \quad (7)$$
$$R^2 = 0.77$$

The response surface analysis (Figure 3.4) showed that lowest trypsin inhibitor activity was obtained at the highest temperature for shortest processing time. As the processing time was increased the trypsin inhibitor activity was decreased at any given temperature. Similar results were observed in lentil, chickpea and pea flours that were thermally processed by boiling in a water bath at $90^0$C for 20 min , which significantly reduced the levels of trypsin inhibitors (Ma et al., 2011). According to the results of Osman *et al.,* the hydrothermal treatment of Tepary bean extract at $100^0$C for 60 min completely inactivated the trypsin inhibitors (Osman et al., 2002). Similar results were observed by Andrade *et al.,* recommending a dry heat of $200^0$C for 20 min for soy flour samples, showing that the temperature plays an essential role in inactivation of trypsin inhibitors (Andrade et al., 2016).

Thus, to obtain soymilk with maximum digestibility with inactivation of trypsin inhibitors, microwave processing (2.45 GHz) at $100^0$C for 8 mins is recommended, in comparison to the conventional thermal processing at $100^0$C for 30 mins.

**Conclusion**

In this study, microwave processing at different conditions of temperature ($70^0$C, $85^0$C and $100^0$C) and time (2, 5 and 8 min) were applied to soymilk samples, and compared to conventional thermal

treatments at the same temperatures and time (10, 20 and 30 min) conditions. The effects of process parameters on trypsin inhibitor and *in-vitro* protein digestibility were investigated. The *in-vitro* protein digestibility increased with increase in treatment time and temperature conditions of the microwave processing ($100^0$C for 8 mins) and the conventional processing ($100^0$C for 30 mins) to 87% and 92% respectively from an initial digestibility of raw soymilk of 80.5%. Similarly the TIA was also dependent on process temperature. The TIA of soymilk after conventional treatment ($100^0$C for 30 mins) was 1% and after microwave processing ($100^0$C for 8 mins), it was 3% that was decreased from an initial TIA of 10% of raw soymilk. The over-processing for the complete elimination of TI's can denature other soy proteins also. Hence the microwave processing can be used as a potential alternative method to the conventional processing of soymilk for the increased digestibility and elimination of anti-nutritional factors.

## CONNECTING TEXT

The functional properties of food proteins are directly influenced by their structure, in turn influences various organoleptic and nutritional properties of the final food product. Molecular dynamics (MD) simulation techniques can be used to understand the structural and conformational changes of protein structures during processing. Soybean trypsin inhibitor protein has an unusual stability and can be inactivated only when the two disulphide bonds present in them are cleaved. The next part of the thesis deals with the application of molecular dynamic (MD) simulations to study the effect of external electric field stress on the conformation and surface properties of soybean trypsin inhibitor (STI) protein. The effect of thermal stress and electric field stress using MD on STI could help us understand the implications of the processing conditions.

## CHAPTER IV

## Effects of Thermal and Electric fields on Soybean Trypsin Inhibitor Protein: A molecular modelling study

### 4.1 Abstract

This study has used molecular dynamics (MD) simulations in studying the unusual stability of Soybean Trypsin Inhibitor (STI) protein. The effects of temperature and oscillating electric fields (0.5 V/nm and 2.45 GHz) have been used to perform simulations using GROMACS software. The conformational changes in the protein were studied using root mean square deviations and secondary structure analysis (STRIDE). It was found that significant rearrangements took place within the protein especially in 'turns' and 'coils', but the core structure was stable under external stresses because of the antiparallel β-sheet structure. This study has also provided evidence that the aromatic residues play a major role in stabilizing the STI protein using Solvent Accessible surface area (SASA) Analysis. Ramachandran plots were used to analyze the stability of the molecules obtained on treatment with temperatures (300 K to 393 K) and oscillating electric fields.

**KEYWORDS:** Soybean trypsin inhibitor; molecular dynamics; GROMACS; Solvent accessible area; Ramachandran plot

## 4.2 Introduction

Proteins are one the most important nutrients present in food apart from carbohydrates and fats, which together are called the major nutrients. These proteins exhibit various functional properties which can be defined as the characteristic physicochemical properties that dictate the protein behaviour in foods during numerous stages of processing and storage. These properties are governed by the changes in the protein structure. Thus, the structure directly influences the functional properties that in turn influences various organoleptic and nutritional properties of the final food product. (Messens, Van Camp, & Huyghebaert, 1997; Vanga, Singh, & Raghavan, 2015b). Various researchers have shown that processing affects the protein structures within the food matrix. From late 70s, both researchers and the food industry have been interested in technology to predict the structural changes of these food proteins that would help engineer high quality food products with superior nutritional output and digestibility, but only with limited success (Nakai, 1983).

During processing, external stresses are applied using thermal (boiling, roasting, infrared heating and dielectric heating) and non-thermal (fermentation, high pressure processing, pulsed electric field and high electric fields) processes. These processes help in increasing the shelf life of food products and also improves their organoleptic properties by producing conformational changes in the protein structure (Vanga, Singh, et al., 2015b). They can also result in modified protein-protein interactions, protein-carbohydrate reactions through Maillard-type reaction contributing to the variation in functional properties of proteins in the final product (Messens, et al., 1997). Therefore, researchers have put more emphasis in understanding the effects of processing on protein structures in recent years (Gomaa & Boye, 2015; Gomaa & Boye, 2013; Mozhaev, Heremans, Frank, Masson, & Balny, 1996; Schulz & Schirmer, 2013; Singh, Vanga, Nair, Gariepy, Orsat, &

Raghavan, 2015). Recently, several techniques including Fourier Transformation Infrared Spectroscopy (FTIR) (Singh, Lahlali, Vanga, Karunakaran, Orsat, & Raghavan, 2015; Vanga, Singh, Kalkan, Gariepy, Orsat, & Raghavan, 2015), Nuclear Magnetic Resonance Imaging (NMR) (Kainosho, Torizawa, Iwashita, Terauchi, Ono, & GÃžntert, 2006), X-ray diffraction (Engh & Huber, 1991; Frauenfelder, Petsko, & Tsernoglou, 1979) and Circular Dichroism (CD) (Provencher & Gloeckner, 1981; Sreerama & Woody, 2000) have been used to study and understand the protein structures. However, they have various limitations depending on the technique used and are very expensive. Moreover, these techniques can only assess the protein structure before and after processing which further complicates the means of evaluating the mechanism involved in protein structural changes in various complex biological structures (Astrakas, Gousias, & Tzaphlidou, 2012; Vanga, Singh, & Raghavan, 2015a).

Molecular dynamics (MD) simulation techniques can act as a viable alternative. It can be applied to overcome the above mentioned issues and can be used to further understand the conformational changes in the protein. The effects of electric fields have been evaluated on chignolin which is the smallest protein stable in solution form using MD simulations. This study concluded that by applying enough external stress the ten residue chignolin protein rotated and aligned itself in the direction of external electric field. With a continuous application of the stress the protein unfolded with changes in the total dipole moment (Astrakas, Gousias, & Tzaphlidou, 2011; Astrakas, et al., 2012). Studies by Wang *et al.,* (2014) showed that the secondary structure of protein (insulin) was intact under the electrical field strength below 0.15 V/nm. Disruptions in the structure were observed on application of electric field strengths of 0.25 V/nm or higher. The results also showed that intensity of the external electric field could either speed up protein folding or destroy the secondary structure of proteins (Wang, Li, He, Chen, & Zhang, 2014). Marracino *et al.,* (2013)

have also used MD simulations in understanding the effects of pulsed and static electric fields on protein folding and unfolding in Myoglobin. They used electric fields in the range of 1 – 10 V/nm and found that fields in the range of 10 V/nm have produced significant secondary structure unfolding and folding (Marracino, Apollonio, Liberti, d'Inzeo, & Amadei, 2013).

In the current study, the secondary structure changes due to external oscillating electric fields and thermal stresses in the soybean trypsin inhibitor will be evaluated. Though, soybean has been traditionally used in Oriental cuisine for many centuries; in North America, it is mainly used as feedstock for animals due to its off flavor (often described as 'beany'). Another important issue with using soybean for human consumption is the presence of various anti nutritional factors like trypsin inhibitors, antivitamins and haemagglutinins (Kwok & Niranjan, 1995). Of the mentioned components, soybean trypsin inhibitor was first found to interfere with digestion and absorption of soybean protein (Kakade, Rackis, McGhee, & Puski, 1974). It was later found that the trypsin inhibitor activity can be reduced by application of moist heat which increases the nutritional value and digestibility of the soymilk and other soybean products (Su & Chang, 2002; Yuan, Chang, Liu, & Xu, 2008). In spite of the advances in processing techniques, research has shown that though the concentration of soybean trypsin can be decreased, it is still persistent in various commercial soy products because of their stability (Spelbrink, Gerrits, Mooji & Giuseppin, 2011; Xiao, Wood, Robertson & Gilani, 2012; Gilani, Xiao & Cockell, 2012). The soybean trypsin inhibitor has a uniquely stable structure because of the large number of β-sheets and has been classified as a 'disordered' antiparallel β-sheet structure (Roychaudhuri, Sarath, Zeece, & Markwell, 2003, 2004). Thus, MD simulation studies on this structure will help us in understanding the effects of external stresses on β-sheet structures.

In this particular study, we are trying to access the secondary structure deviations in the soybean trypsin inhibitor due to thermal and oscillating electromagnetic fields (microwave/radio frequency processing) which is necessary to understand the influence of the processing methods on an atomic and molecular level as mentioned above. The quantification of the conformational changes will be done using root mean square deviations and STRIDE analysis at various levels of simulation parameters (Singh, Orsat, & Raghavan, 2013). Surface properties like hydrophobicity and hydrophilicity will also be assessed in this study. Ramachandran plots have been used to validate the protein structures obtained from the simulations.

## 4.3 Materials and Methods

### 4.3.1 Molecular modeling

All MD simulations were performed using the classical MD algorithm as implemented in Groningen machine for chemical simulations (GROMACS) software package, version 4.5.5 from the Stockholm Center for Biomembrane Research, Stockholm, Sweden (Hess, Kutzner, Van Der Spoel, & Lindahl, 2008). The Kunitz-type trypsin inhibitor (KTI) protein from soybean with PDB accession code 1AVU (Song & Suh, 1998) was downloaded. This KTI protein molecule downloaded consists of 181 amino acids residues and two disulphide bonds between cysteine residues 39-86 & 136-145. The CHARMM27 (Astrakas, et al., 2011) and TIP3P water model (Jorgensen, Chandrasekhar, Madura, Impey, & Klein, 1983) were adopted for protein and solvent, respectively.

The protein was placed in a periodic cubic water box of dimensions 11.005 X 11.005 X 11.005 (nm) to satisfy the minimum image convention. Subsequently, 43137 water molecules were filled in the box. The entire system was neutralized using seven sodium atoms. The system was first

energy minimized with converging criterion of maximum force value of 10 kJ/nm/mol using steepest descent for 20000 steps to remove close contacts. Then, two 100 ps equilibrations were carried out at the constant temperature, constant volume (NVT) ensemble and at the constant temperature, constant pressure (NPT) ensemble, successively.

A total of eight MD simulations were run at five nanoseconds to evaluate the effect of temperature (300 K, 343 K, 373 K and 394 K MD) and oscillating external electric field at an intensity of 0.5 V/nm with a frequency of 2.45 GHz. For comparison, MD simulations in the absence of an external electric field at the same temperatures were carried out (Vanga, Singh, & Raghavan, 2015a). All the electric fields were applied at the X axis of the equilibrated solvated protein system. During the MD simulation, the temperature was maintained using Berendsen thermostat (Berendsen, Postma, van Gunsteren, DiNola, & Haak, 1984) and pressure was set to one bar using the Parrinello- Rahman barostat (Parrinello & Rahman, 1980). The electric field conditions were selected to simulate microwave assisted processing conditions (0.5 V/nm and 2.45 GHz). All the data were analyzed by GROMACS analyzing tools. VMD software was utilized to draw structural diagrams (Humphrey, Dalke, & Schulten, 1996).

### 4.3.2 Ramachandran plot

It is well known that the stability of any protein is greatly dependent on the two torsion angles in any polypeptide chain that influence the rotation of the protein backbone structure. These angles are called Ramachandran angles which are used to plot the Ramachandran plot. The first angle is the Phi-φ angle (between N-Cα) and second is Psi-ψ angle (between Cα-C) (Ramachandran, 1963; Ramakrishnan & Ramachandran, 1965). The trajectory file of the simulated molecule between the frames 4998 and 5000, i.e. structural conformation at 4.99 ns, which were obtained using the GROMACS internal functions have been used for the analysis. The Ramachandran plots and the

data associated regarding the residues in favoured regions have been generated using the MolProbity software (Chen, et al., 2009; Davis, et al., 2007)

## 4.4 Results and Discussions

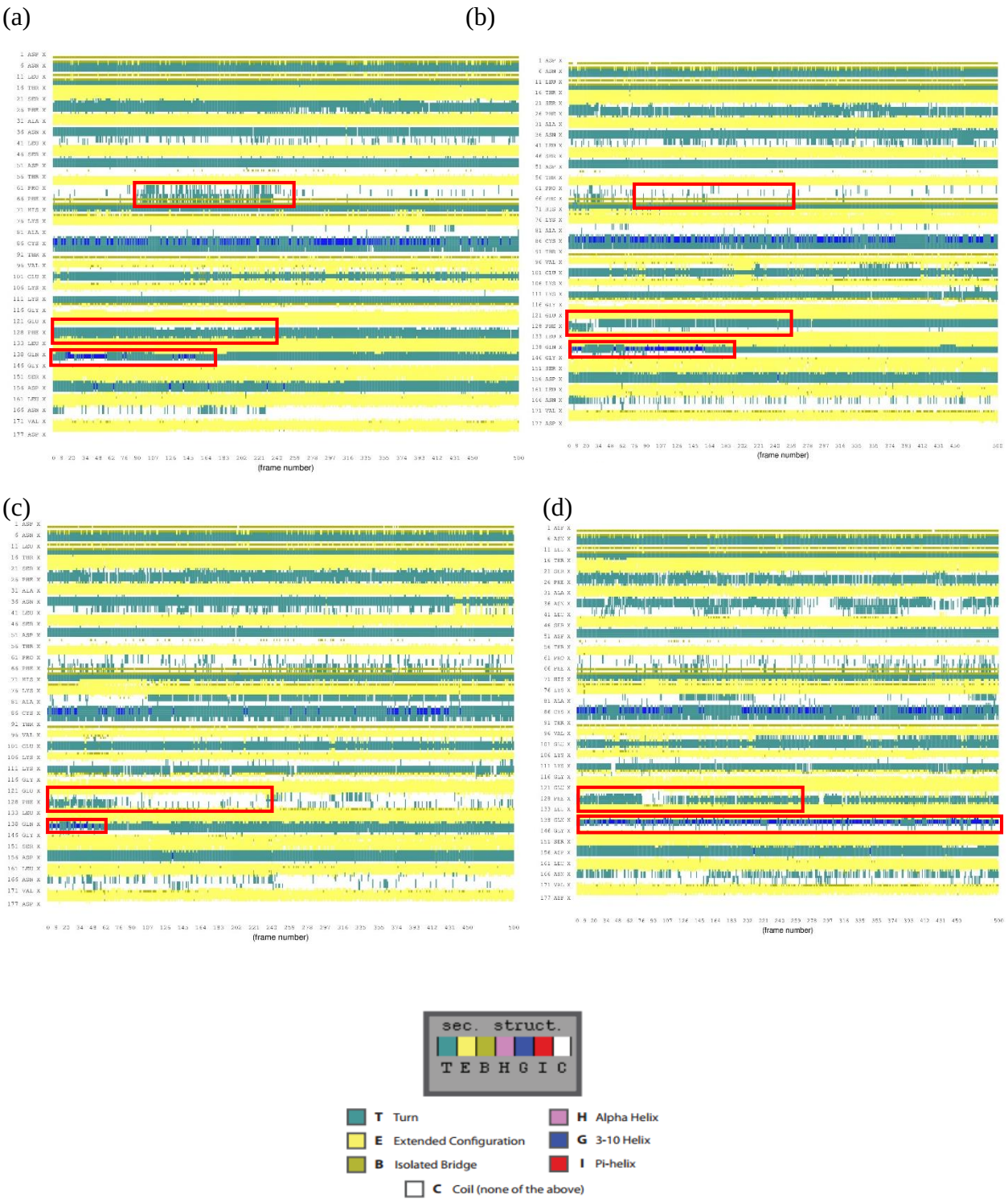
### 4.4.1 Secondary Structure Analysis

The secondary structure analysis was conducted using the STRIDE algorithm (Frishman & Argos, 1995) provided within the VMD software (version 1.9.1) (Humphrey, et al., 1996). The stride algorithm uses the torsional angle information and the hydrogen bond energies generated in the background upon running the PDB file coordinates to map and assign the most probable secondary structure to each residue (Frishman, et al., 1995; Heinig & Frishman, 2004). Soybean Trypsin Inhibitor (STI) which is classified as Serine protease inhibitor with PDB ID 1AVU was used for the simulations in this study (Berman, et al., 2000; Song, et al., 1998). This molecule contains a total of 181 amino acid residues with 1% of the secondary structure as 3/10 helix and 43% as beta sheets (extended configurations). A total of 79 residues (73 according to DSSP) conform to a beta sheet structure. The other secondary structures observed include turns and coils. But, it has to be noted that the STI molecule does not contain any alpha helical secondary structures but the only one helical structure which is the 3/10 helix observed between residues 86-88 (M-C) (Frishman, et al., 1995). Beta sheets are observed between the following residues 4-5 (L-D), 9-10 (N-P), 15-21 (G-S), 29-32 (I-A), 42-45 (T-Q), 56-59 (T-I), 74-77 (S-F), 94-96 (S-V), 104-106 (A-K), 113-114 (A-M), 116-122 (G-R), 131-137 (Y-P), 146-152 (G-I), 159-164 (R-S), 171-175 (V-K) (Frishman, et al., 1995; Kabsch & Sander, 1983).

STRIDE analysis is a powerful tool to find the changes in these secondary structures during the simulation under the external stresses of different orders. The output in VMD is given in form of

a chart with residues on the Y axis and the time/frames on X axis as shown in the Fig.4.1 & 4.2. Each colour band corresponding to the residues represent the secondary structure orientation of the residue with respect to the duration of simulation. They aid in finding out the residues participating in the secondary structure deviations. From Figures 4.1 & 4.2, it is clear that STI is quite stable under the application of external stress, especially due to the presence of disulphide bonds between the Cysteine residues 39-86 & 136-145 (Cabrera-Orozco, Jiménez-Martínez, & Dávila-Ortiz, 2013).

**Figure 4.1** STRIDE analysis showing the deviations in 1AVU with no electric field (a) 300 K (b) 343 K (c) 373 K (d) 394 K



Comparing Figure 4.1(a) & (b), shows that the external stresses caused significant disruptions between residues 61 - 66 (frame 90-240) with increase in temperature from 300 K to 343 K. As the temperature increased further, higher amount of deviations in turn structures were observed present in the core of the molecule between the residues 76-81. During the same time, the turns that were present between the residues 121-128 in Fig. 4.1(a) and (b) are absent in Fig. 4.1(c). This could be a result of relocation of the secondary structures within the core structure. Further, 3/10 helix between 86-88 became highly unstable and the residues have started to exhibit characteristics of turns with increase in temperature from 300 K to 393 K. As the temperature increased from 373 K (Fig. 4.1(c)) to 394 K (Fig. 4.1(d)) the turns between residues 76-81 were relocated back to 121-128. There were further disruptions between 31-41 residues which were mostly stable in the simulations with temperatures between 300 K and 373 K. A 3/10 helix also appeared between the residues 138-141 for the whole length of simulation at 394 K. Fig. 4.2 shows the secondary structure changes in STI when an oscillating field of 0.5 V/nm and 2.45 GHz was applied. One of the significant secondary structure changes was observed as the temperature increased from 300 K to 394 K is between the residues 36-41. In this region, the number of turns reduced with an increase in temperature. Another important observation to note is between the residues 138-141. Upon application of external oscillating field they showed characteristics of 3/10 helix at 300 K; but as the temperature increased further, the duration for which these residues showed helical secondary structure characteristics reduced.

### 4.4.2 Root Mean Square Deviation (RMSD) Analysis

Root mean square deviation is an important tool used in predicting the arithmetic values of the deviations in the atoms in comparison to a reference molecule caused by the external stress (Budi,

S. Legge, H. Treutlein, & I. Yarovsky, 2004; Vanga, Singh, & Raghavan, 2015a). The RMSD is calculated by using the following equation (Singh, Orsat, et al., 2013).

$$RMSD = \sqrt{\frac{1}{N}\sum_{i=1}^{N} |r_{final}(i) - r_{initial}(i)|^2} \quad (1)$$

Where $r_{final}$ ($i$) is the final coordinates of an atom $i$, and *rinitial* ($i$) is the initial coordinate of the atom $i$, and $N$ is the number of atoms.

**Table 4.1:** RMSD values obtained for 1AVU protein (Soybean trypsin inhibitor)

| Temperature (K) | No Electric Field (nm) | Oscillating Electric Field (0.5 V/nm, 2.45 GHz) (nm) |
|---|---|---|
| 300 | 0.203933 ± 0.0181 | 0.182374 ± 0.0131 |
| 343 | 0.242804 ± 0.0279 | 0.221049 ± 0.0205 |
| 373 | 0.230848 ± 0.0179 | 0.222425 ± 0.0141 |
| 394 | 0.216849 ± 0.0155 | 0.235596 ± 0.0167 |

Table 4.1 summarizes the average RMSD values observed throughout the simulation of the STI molecule. It is quite clear that the RMSD values obtained in the presence of oscillating electric field are much lower compared to that of zero electric field, especially at 300 K. Similar results were observed by Vanga *et al.*, (Vanga, Singh, & Raghavan, 2015a), where Ara h 6 was used for simulation. As the temperature increased further to 343 K the RMSD increased significantly in both cases (with and without oscillating electric field).

**Figure 4.3 (a):** RMSD deviations observed in 1AVU with only temperature and no external electric field

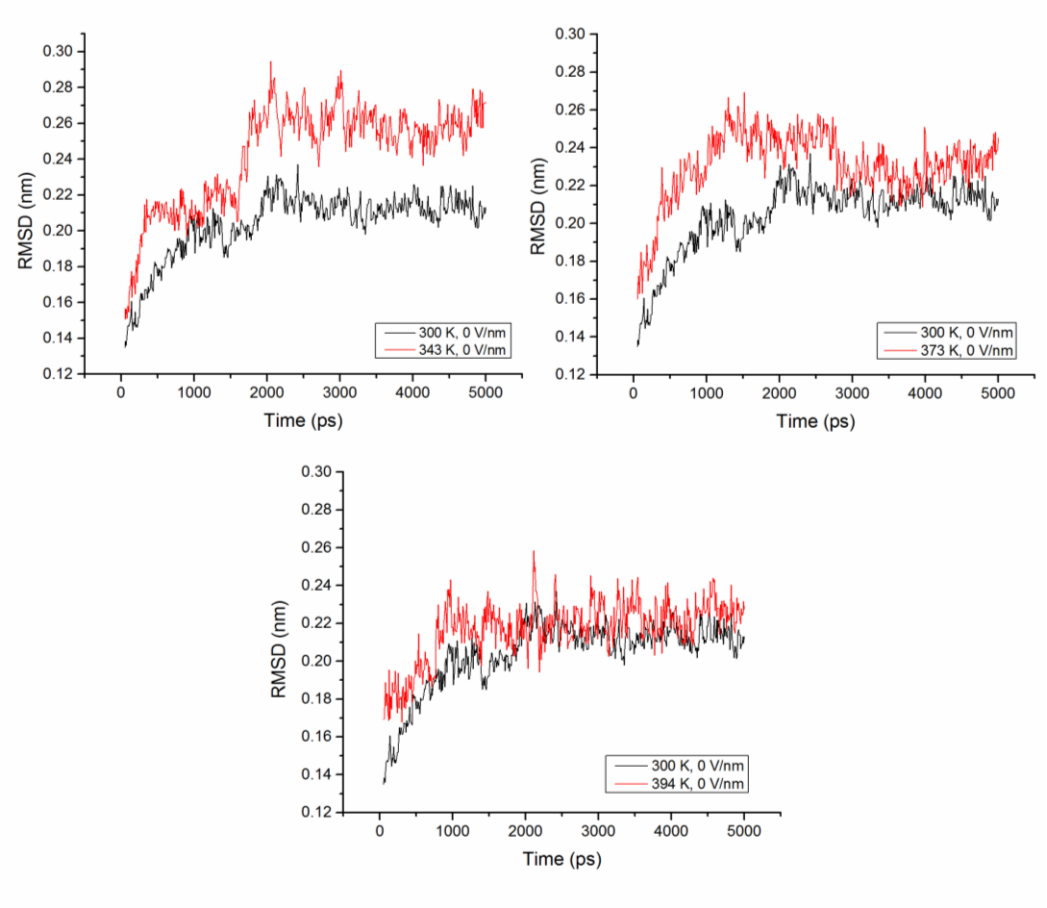


**Figure 4.3 (b):** RMSD deviations observed in 1AVU with temperature and an oscillating external electric field of 0.5 V/nm, 2.45 GHz

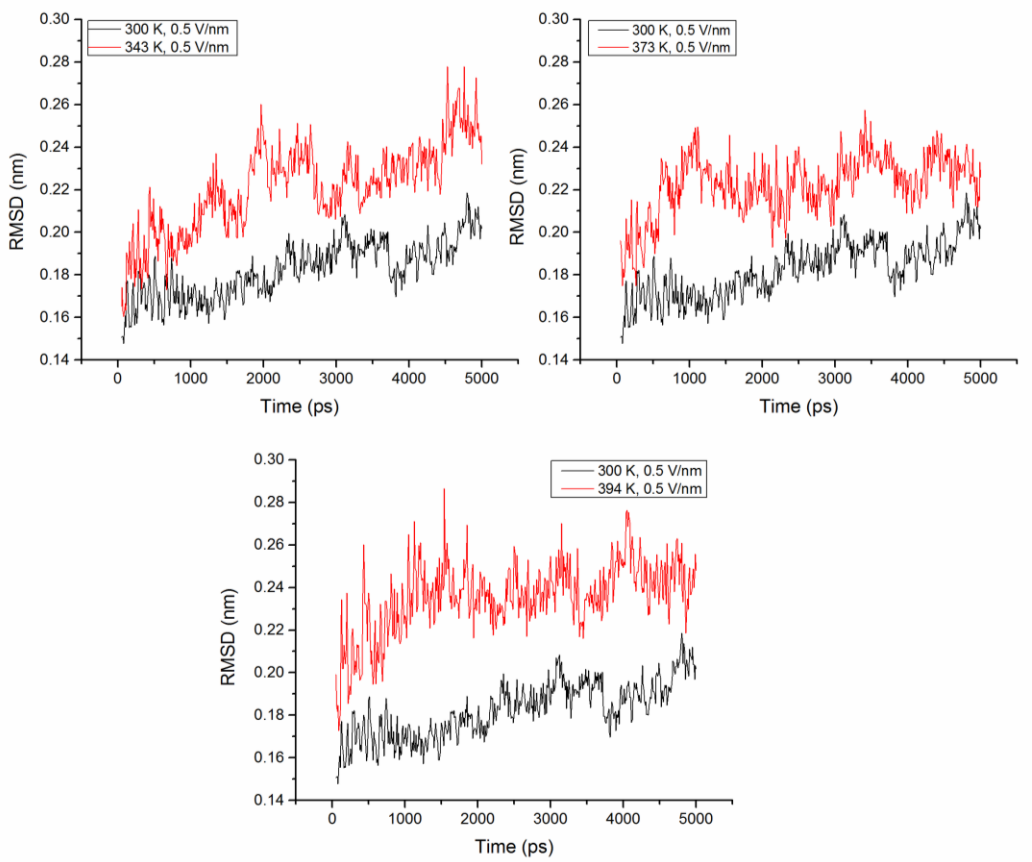



Fig. 4.3(a) shows the trend in RMSD with increased temperature in comparison to the simulation carried out at 300 K. It is interesting to note that the RMSD values observed at 394 K are lower compared to the values at 343 K and 373 K when no oscillating field is applied. When an oscillating field of 0.5 V/nm at 2.45 GHz is applied, a significant jump in the RMSD values is observed between 300 K and 343 K. Further increase in temperature resulted in almost similar of

RMSD. Similar trend is observed in Table 4.1 where the RMSD values obtained at higher temperatures are higher than those obtained at room temperature (~300 K). Similar observations in RMSD values between no electric field and oscillating electric field reinforces the idea that the processing treatment does not affect the stability of the STI significantly within the time frame of five nanoseconds

### 4.4.3 Solvent Accessible Surface Area (SASA)

Surface properties play an important role in determining the interactions between the protein and various other molecules and solvents; and thus are important in determining the characteristic functions of the protein. These surface properties are highly dependent on the structural conformations with the protein and even slight changes to these conformations can result in change of functional properties of the protein (Singh, Munshi, & Raghavan, 2013; Wellner et al., 2005). SASA is estimated by using in built commands in the GROMACS software. It can be defined as an estimate of the surface area that is available for contact with solvents and other molecules. It is calculated by using the following equation (Lee & Richards, 1971; Vanga, Singh, & Raghavan, 2015a)

$$SASA = A = \sum (R / \sqrt{R^2 - Z_i^2}) \times D \times L_i \qquad (2)$$

Where, A is the surface area, R is the radius of atom, $L_i$ is the length of the arc drawn on a given section *i*; $Z_i$ is the perpendicular distance of section *i* from the centre of the sphere. (Note: Lee and Richards estimated SASA by only considering the static accessibility, but it does not account for the potential flexibility of the structure).

**Table 4.2:** Solvent accessible surface area for 1AVU protein (Soybean trypsin inhibitor)

| Temperature (K) | No Electric Field (nm) | Oscillating Electric Field (0.5 V/nm, 2.45 GHz) (nm) |
|---|---|---|
| 300 | 95.73 ± 1.8928 | 97.34 ± 1.3557 |
| 343 | 99.89 ± 1.7104 | 98.12 ± 1.6790 |
| 373 | 97.34 ± 1.5006 | 97.63 ± 1.6276 |
| 394 | 97.67 ± 1.4959 | 97.34 ± 1.5351 |

Table 4.2 estimates the average SASA values for the 1AVU STI molecule during simulation with and without the oscillating electric field. When the temperature is increased from 300 K to 343 K a significant rise was observed in the SASA values which is a clear indicator that new binding sites were made available on the surface of the molecule (Fig. 4.4(a)). On further increasing the temperature, the SASA values remained almost constant. With the duration of simulation the surface areas at temperatures 373 K and 394 K remained constant without significant deviations as observed in Fig. 4(a). It is interesting to note that the SASA for the oscillating electric field at 300 K is slightly higher than that of the values obtained without application of the electric field. Fig. 4.4(b) shows the trend in SASA during the simulation. It is quite clear that oscillating electric field has caused an increased external stress and thus the protein is more volatile to movement throughout the simulations irrespective of temperature. These fluctuations might be due to the oscillating field applied which is forcing the amino acid residues to move according to the direction or orientation of the field. But, the STI molecule is stable without any major deviations in the overall SASA in spite of the oscillating electric field within five nanoseconds. This was not the case in the studies conducted on Insulin (Budi, Legge, Treutlein, & Yarovsky, 2007; Budi, Legge, Treutlein, & Yarovsky, 2004), Myoglobin (Marracino, et al., 2013) and Ara h 6 allergen (Vanga, Singh, et al., 2015a) which showed a significant effect on the secondary structures upon the application of electric fields.

**Figure 4.4(a):** Solvent Accessible Surface Area (SASA) values observed in STI with only temperature and no external electric field

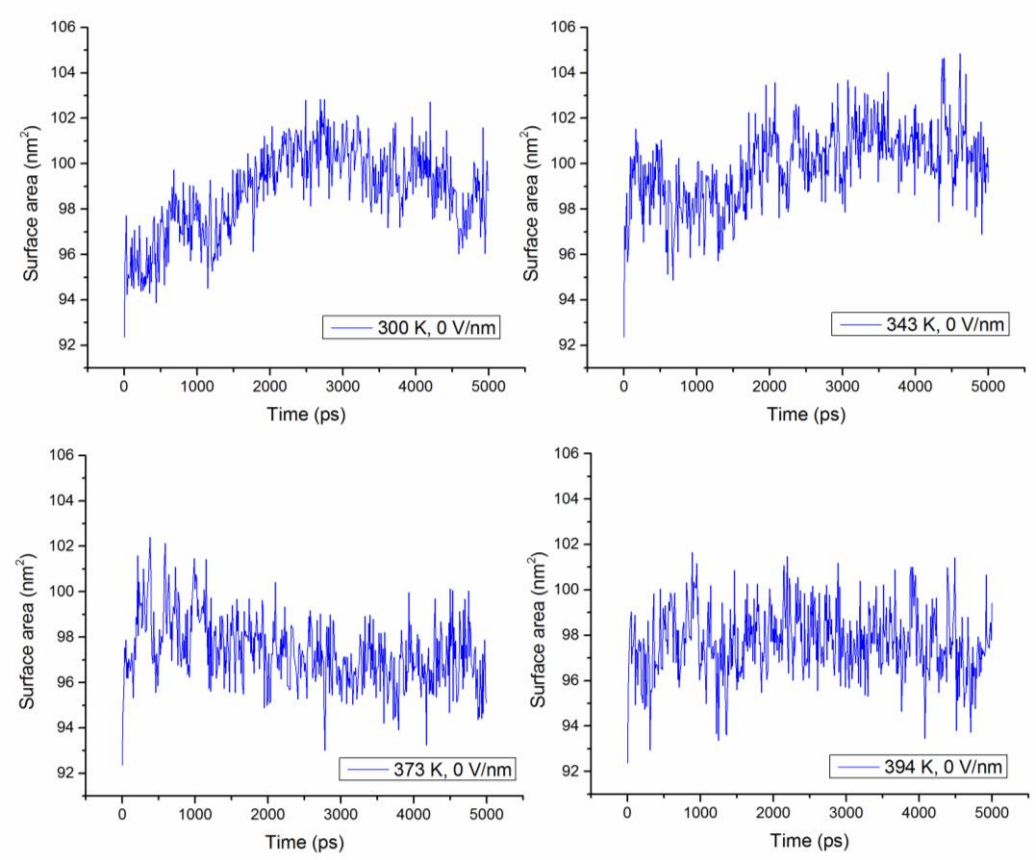
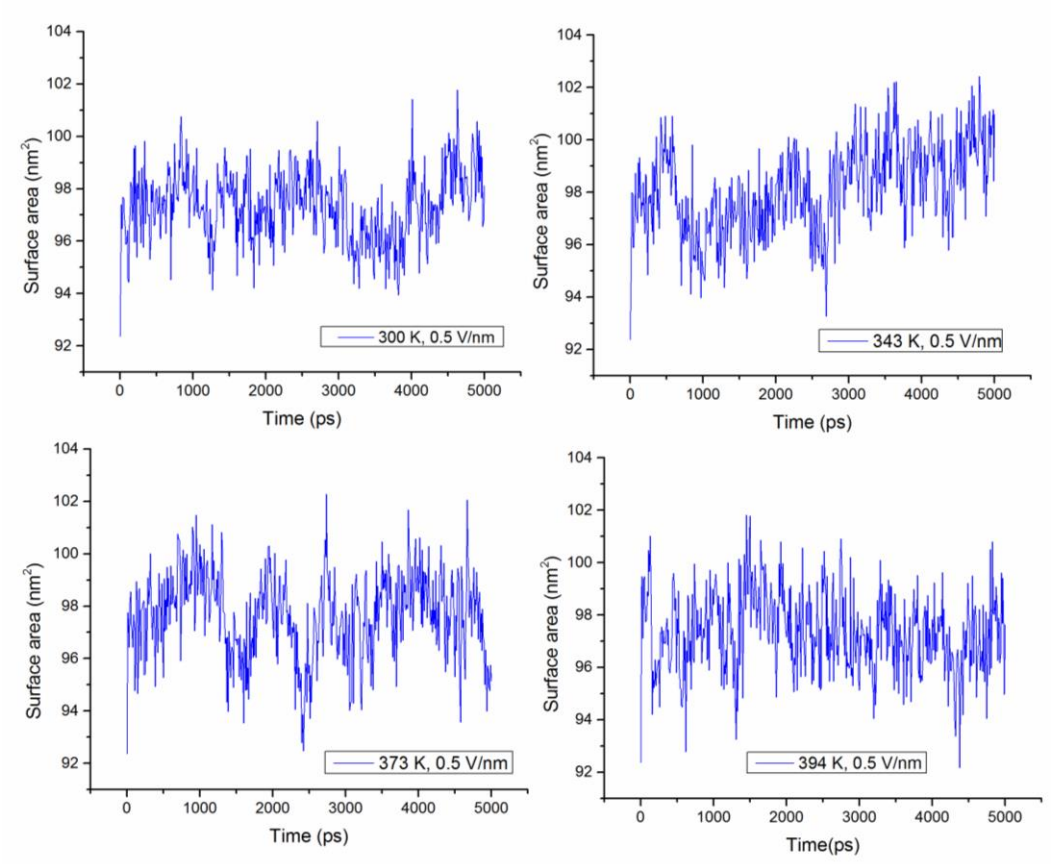


**Figure 4.4(b):** Solvent Accessible Surface Area (SASA) values observed in STI with an oscillating external electric field of 0.5 V/nm, 2.45 GHz.




One of the main reasons for this unusual stability of the STI protein to oscillating field can be attributed to the absence of alpha helices in the molecule. Because, the alpha helices present within the protein structure have their own dipole moment; and thus under the presence of an external electric field, these helices would try to orient themselves which causes immense stress in the residues forcing them to change the conformational structure (Budi, et al., 2004; Todorova, Legge, Treutlein, & Yarovsky, 2008; Vanga, Singh, et al., 2015a). But, due to the absence of alpha helices and a packed core structure with multiple beta sheets the STI molecule has a higher stability and

re-folding characteristics (Roychaudhuri et al., 2003, 2004). The presence of two disulphide bonds between Cys 39–Cys 86 and Cys 138–Cys 145 is also another contributing factor to the stability of the molecule.

**Table 4.3:** Solvent accessible areas of selected residues present in the STI protein

| **Residues** | **1AVU** | **No electric Field (Å²)** | | | | **Oscillating electric field (Å²)** | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **(Å²)** | **300 K** | **343 K** | **373 K** | **394 K** | **300 K** | **343 K** | **373 K** | **394 K** |
| **Trp – 93** | **9.1** | 12.5 | 10.7 | 8.7 | 6.5 | 19.4 | 10.1 | 12.6 | 14.8 |
| **Trp – 117** | **90.7** | 119.8 | 108.3 | 96.7 | 91.8 | 107.7 | 131.2 | 78.0 | 72.1 |
| **Cys – 39** | **22.3** | 12.4 | 13.3 | 2.2 | 22.2 | 16.5 | 5.1 | 20.3 | 36.2 |
| **Cys – 86** | **1.9** | 1.8 | 5.4 | 2.0 | 10.6 | 4.5 | 4.3 | 16.9 | 11.7 |
| **Cys – 138** | **21.1** | 13.7 | 19.6 | 0.2 | 6.4 | 22.8 | 10.1 | 0.2 | 5.2 |
| **Cys – 145** | **29.2** | 24.8 | 56.1 | 38.8 | 41.2 | 70.9 | 47.2 | 46.8 | 65.5 |

In 2003, studies by Roychaudhuri *et al.,* have showed that the aromatic amino acid residues especially tryptophan present within the structure play a critical role in determining the unfolding and refolding characteristics of the STI molecule (Roychaudhuri et al., 2003). They observed that the solvent accessibility of the tryptophan has increased which are actually present within the core hydrophobic structure with rise in the temperature resulting in the unfolding of STI secondary structure. We have used STRIDE software (Heinig & Frishman, 2004) to predict the solvent accessible areas of STI proteins that have been exposed to temperatures between 300 K and 394 K and oscillating electric fields of 0.5 V/nm at 2.45 GHz frequency. The values that are predicted according to Eisenhaber *et al.,* have are reported in Table 4.3 (Eisenhaber & Argos, 1993; Eisenhaber, Lijnzaad, Argos, Sander, & Scharf, 1995). It is evident from this table that the processing has resulted in higher SASA values in Trp residues of the STI protein. Especially between the temperatures of 343 K and 300 K both with and without the application of oscillating electric fields. But, on further increasing the temperature to 394 K, the SASA values reduced in

the absence of the electric field and increased in the presence of the oscillating electric fields which further supports the assumption that the oscillating dipole is causing the residues to reorient themselves in the direction of the applied field.

The SASA values of cysteine residues forming disulphide bridges have also been tabulated (Table 4.3). Tetenbaum et al., have mentioned that these residues are on the surface of the protein and thus are highly accessible to various solvents and other molecules (Tetenbaum & Miller, 2001). Several experiments have revealed that these disulphide bonds can be reduced at over 313 K when provided with proper conditions and this reduction follows a non-cooperative path and are reversible. The 1AVU column reiterates this point that these cys residues are indeed solvent accessible where 75% of them have a solvent accessible area of over 20 $Å^2$. This area further increased with temperature, especially cys-86 and cys-145. The SASA increased five folds in the case of cys-86 residue both in the presence and absence of the oscillating electric field. This increase in SASA of cys residues can directly influence the disulphide bridge stability as it would provide access for external solvents and enzymes to act on the protein. And thus, reduction of these disulphide bonds could result in collapse of the secondary structure of STI and change the interactive characteristics with solvents and enzymes (Lehle, Wrba, & Jaenicke, 1994; Steiner, De Lorenzo, & Anfinsen, 1965; Tetenbaum & Miller, 2001). This data further supports that the combination of temperature and oscillating electric fields can have a higher effect in destabilizing the residues and varying the surface properties which can directly influence the functional properties of any protein molecule.

### 4.4.4 Ramachandran plot Analysis

Ramachandran plot has been traditionally used to validate protein structures for over 50 years now with continuous modifications to increase the accuracy of the predictions. In 1963, Ramachandran

*et al*., illustrated how certain combinations of φ-ψ torsion angles would cause steric interference in protein structures making certain combinations unattainable in practice. This steric interference is due to the clashes between protein backbone residues and/or side chains which make up the 'disallowed' region on the 2D plot produced using the φ-ψ torsion angles. The remaining regions are the 'allowed' region - each correspond to a particular secondary structure (Ramachandran, Ramakrishnan, & Sasisekharan, 1963). For analysis of the simulated protein structures, MolProbity online software is used (Chen et al., 2009; Davis, Murray, Richardson, & Richardson, 2004). In general, a Ramachandran plot generates 4 different plots: Proline, Pre-proline, Glycine and all the other residues. Proline and Glycine play a unique role in determining the secondary structure conformations and hence are plotted separately. Further, the proline also influences the residues preceding it in the protein chain and hence are plotted on a separate plot. These groups of residues i.e. proline, glycine and pre-proline show a different characteristic φ-ψ torsion angles (Ho & Brasseur, 2005; MacArthur & Thornton, 1991). MolProbity is used for this analysis because it uses a total of 100,000 residues from about 500 high resolution protein structures to obtain the contours on the plot (Lovell et al., 2003). After recent upgrades, the MolProbity software has separated the plots for residues Isoleucine and Valine from general plots and further divided the Proline plots into two (separate for cis-Proline and trans-Proline) causing the six final Ramachandran plots for each analysis.. The contours obtained in this analysis are divided into 2 regions: Inner region - Favoured (shown in light blue in Fig.4.5) which constitutes 98% of the residues and the outer region - Allowed (shown in dark blue in Fig.4.5) consisting 99.8%. The remaining region outside these blue lines is for the Outlier residues. The data set used for analysis has also increased after the upgrade to a total of 1.5 million residues obtained from over 8000 high resolution structures (Laskowski, Furnham, & Thornton, 2013).

**Figure 4.5:** Ramachandran plot of 1AVU protein generated using MolProbity

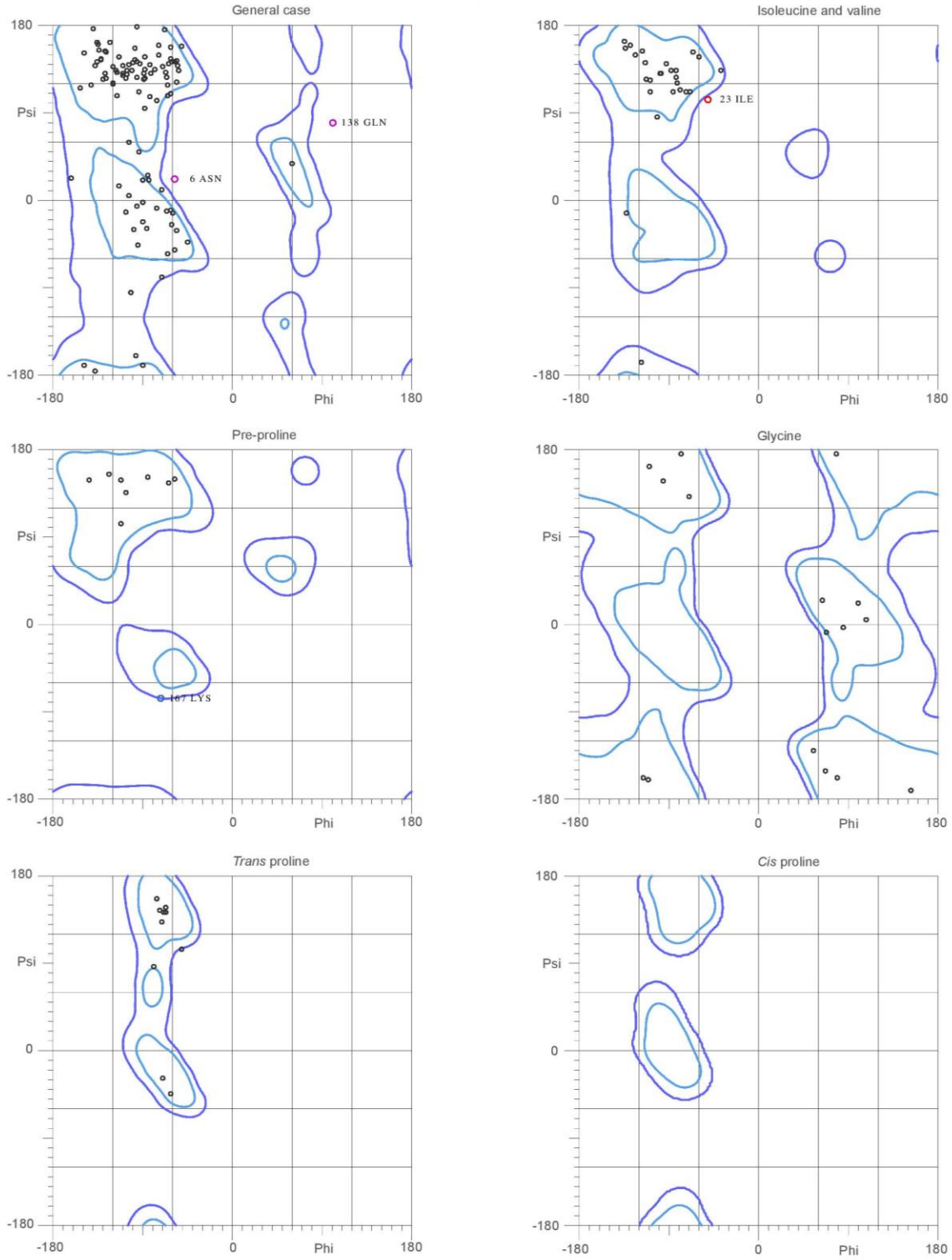
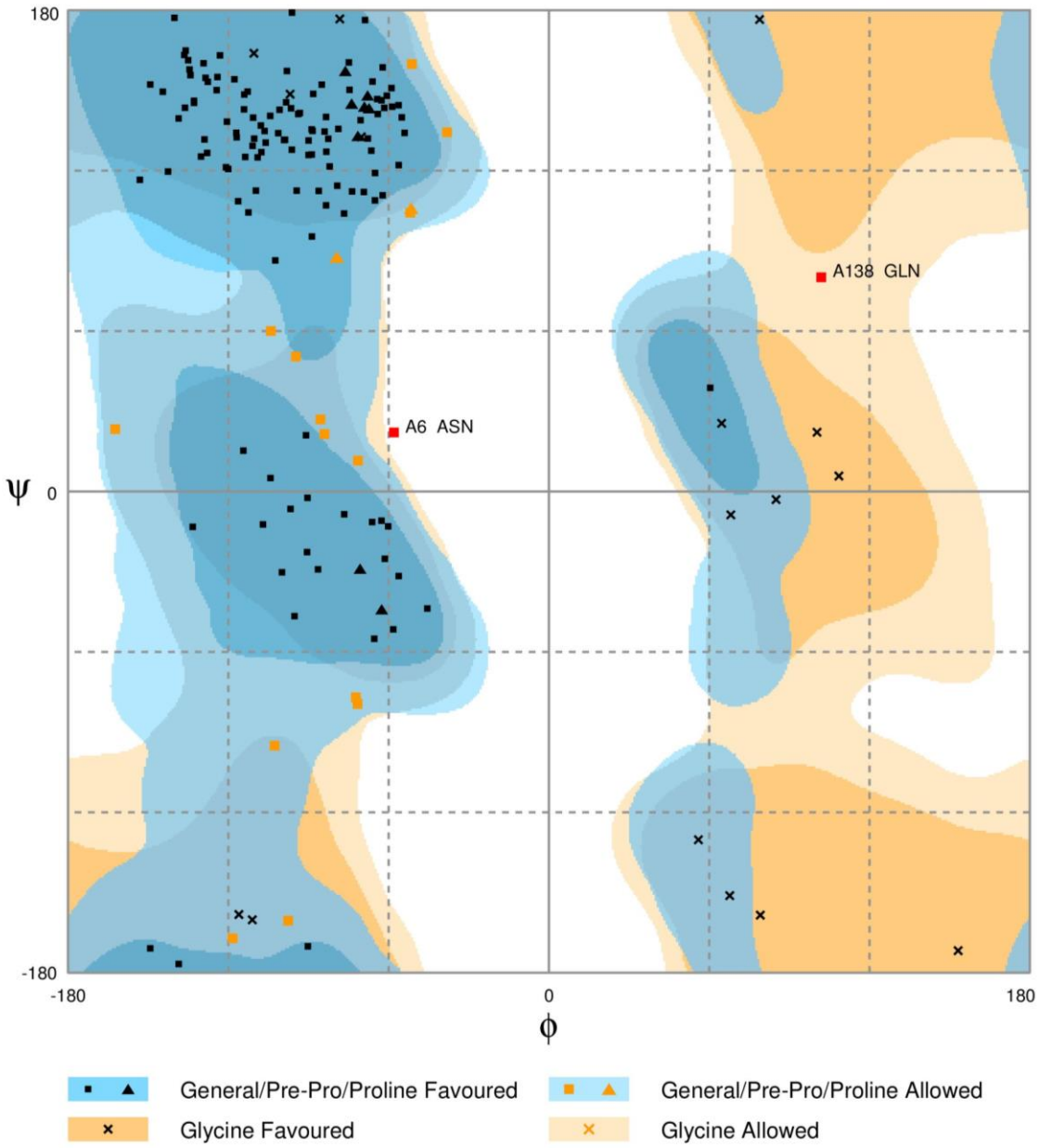


**Figure 4.6:** Ramachandran plot generated for the STI protein using the RAMPAGE software which generates a combined plot with different shades showing the favoured and allowed regions




| | |
|---|---|
| Number of residues in favoured region (~98.0% expected) | : 148 (89.2%) |
| Number of residues in allowed region (~2.0% expected) | : 16 (9.6%) |
| Number of residues in outlier region | : 2 (1.2%) |

Fig.4.5 plots the φ-ψ torsion angles of the STI molecule in six Ramachandran diagrams using the software MolProbity with φ (Phi) on X-axis and ψ (Psi) on Y-axis. Each of the six plots have different contours that are generated to show the favoured and allowed regions on analysing the high resolution structures obtained experimentally. The first one is the general case where majority of the amino acids fall in the favoured and allowed regions of the plot. Two residues (6-Asn and 138-Gln) are marked in purple to represent that they are outliers i.e. their current φ-ψ torsion angles would cause steric interference within the simulated molecule. The second plot on right is for Isoleucine and Valine which shows one outlier. Similarly, Pre-proline, Glycine, trans-proline and cis-proline plots follow which all show the residues in the favoured and allowed regions. Similar plots have been made for the final structure attained by the STI protein upon simulation using only temperature and combination of temperature and oscillating electric fields (supplementary material Fig A & Fig. B). Fig. 4.6 is also a Ramachandran plot generated for the STI protein using the RAMPAGE software which generates a combined plot with different shades showing the favoured and allowed regions (Lovell et al., 2003).

Tables 4.4(a), (b) & (c) outline the statistical data generated after the analysis which shows the total percent of residues that fall in the favoured, allowed and outlying regions of the Ramachandran plot.

**Table 4.4(a):** Model Validation reports of STI molecule

| Parameters | Goal | 1AVU |
|---|---|---|
| **Ramachandran Favoured** | Maximize | 86.1% |
| **Ramachandran allowed** | Maximize | 97.6% |
| **Ramachandran Outliers** | Minimize | 2.4% |
| **Cβ deviations (>2.5 nm)** | Minimize | 0% |
| **Bad bonds** | Minimize | 0% |
| **Bad angles** | Minimize | 0% |

**Table 4.4(b):** Model Validation reports of STI molecule simulated without electric fields

| Parameters | Goal | 300 K | 343 K | 373 K | 394 K |
|---|---|---|---|---|---|
| **Ramachandran Favoured** | Maximize | 95.3% | 93.5% | 94.7% | 89.4% |
| **Ramachandran allowed** | Maximize | 98.8% | 96.5% | 98.8% | 97.1% |
| **Ramachandran Outliers** | Minimize | 1.2% | 3.5% | 1.2% | 2.9% |
| **Cβ deviations (>2.5 nm)** | Minimize | 8.33% | 10.9% | 15.38% | 16.03% |
| **Bad bonds** | Minimize | 0.07% | 0% | 0.07% | 0.07% |
| **Bad angles** | Minimize | 4.51% | 5.47% | 7.24% | 6.28% |

**Table 4.4(c):** Model Validation reports of STI molecule simulated with oscillating electric fields at 0.5 V/nm at 2.45 GHz

| Parameters | Goal | 300 K | 343 K | 373 K | 394 K |
|---|---|---|---|---|---|
| **Ramachandran Favoured** | Maximize | 97.1% | 91.2% | 91.2% | 91.2% |
| **Ramachandran allowed** | Maximize | 97.6% | 98.2% | 96.5% | 98.8% |
| **Ramachandran Outliers** | Minimize | 2.4% | 1.8% | 3.5% | 1.2% |
| **Cβ deviations (>2.5 nm)** | Minimize | 7.64% | 16.03% | 12.18% | 17.31% |
| **Bad bonds** | Minimize | 0% | 0.07% | 0.15% | |
| **Bad angles** | Minimize | 4.13% | 5.53% | 5.85% | |

It should be noted that the percentage shown corresponding to the allowed regions includes the residues that are in the favoured region because the favoured region is an intrinsic part of the allowed region. This table (Table 4.4) also outlines the bad bonds and bad angles throughout the protein backbone which deals with deviations in the ideal geometrical parameters. They are often caused due to deviations in the local structure from the idealistic values and are helpful in performing diagnostics on the local structure. The $C^{\beta}$ (beta-carbon) deviations are a result of the overall misfitting caused due to bad bonds and angles and distortions in dihedrals (Chen et al., 2009). The STI molecule (PDB ID: 1AVU) downloaded from the PDB website is obtained from X-ray diffraction at resolution of 2.3 Å (Song & Suh., 1998a). From Table 4.4(a) it is quite clear that this structure is free of bad bonds and bad angles and thus no $C^{\beta}$ deviations. The 'Goal' column

in the table gives us the general idea of the trend that the corresponding values are followed for attaining a highly stable structure. This molecule has about 86.1% of the residues in the favoured region and a total of 97.6% in allowed regions with only 2.4% as outliers.

The PDB files of the final conformation attained by the STI protein when treated with only temperature have been used to generate the data in Table 4.4(b). The number of residues in the favoured regions increased compared to the original structure (86.1%) with highest value of 95.3% (300 K) and lowest value of 89.4% (393 K). The % outliers also reduced significantly in case of the protein structures obtained after simulations at 300 K and 373 K. The total number of residues in the allowed regions were over 95% in all the cases (least was 96.5% for 343 K) which means that the simulation yielded stable structures without many dislocations and misfittings. Bad bonds were recorded to be 0.07% (1 in 1374 bonds) which also proves that the local misfittings are minimal. But, bad angles have increased significantly from 0% as shown in Table 4.4(b). This in turn effected the $C^{\beta}$ deviations which are also raised to 8.33% - 16.03%, increasing with rise in temperature. This is common as the simulated structures will have higher deviations from the original structure due to the nature of prediction and the force fields used in the simulation. In the case of the simulated structures obtained in the presence of the oscillating electric fields as shown in Table 4.4(c), the residues present in the favoured regions although greater than the original structure, they are however is significantly smaller compared to the values of Table 4.4(b). But, the overall percentages of the residues in allowed regions and in disallowed regions (outliers) remains almost the same. It is generally assumed that the protein structures generated through simulations have a lower number of residues in the favoured and allowed regions. But, this is not the case with our simulations because before simulation, the STI molecule goes through an energy minimization which refines the PDB protein structure further (Dehury et al., 2015).

**Table 4.5:** List of Outlier residues in the Ramachandran plots generated using MolProbity

| Parameter | Temperatures | List of Ramachandran Outliers (phi,psi) |
|---|---|---|
| **1AVU** | | 6 ASN (-58.0, 22.1); 23 ILE (-51.9, 104.3); 138 GLN (101.9, 80.1); 167 LYS (-72.3, -77.0) |
| **No Electric Field** | 300 K | 128 PHE (129.0, 63.5); 169 LEU (107.8, 107.8) |
| | 343 K | 40 PRO (-101.7, 78.5); 84 MET (-52.4, 5.4); 98 ASP (-29.8, 136.1); 123 VAL (-38.9, 163.2); 128 PHE (108.6, -166.8);168 PRO (-89.7, -35.5) |
| | 373K | 128 PHE (104.3, 79.9); 139 GLN (79.8, 53.1) |
| | 393 K | 35 GLY (-176.1, -11.7); 40 PRO (-107.9, 86.0); 84 MET (72.7, -96.5); 124 SER (46.7, 82.8); 127 GLU (167.4, 116.1) |
| **Oscillating Electric Field (0.5 V/nm, 2.45 GHz)** | 300 K | 40 PRO (-105.9, 83.0); 98 ASP (82.9, 46.6); 128 PHE (109.8, 92.0); 169 LEU (122.9, 103.4) |
| | 343 K | 40 PRO (-40.6, 98.0); 128 PHE (131.7, 174.3); 137 PRO (-102.7, 119.9) |
| | 373K | 84 MET (51.5, -92.6); 112 ASP (-68.7, 20.3); 123 VAL (-156.1, -9.5); 128 PHE (117.5, 177.2) 139 GLN (74.3, -78.5); 169 LEU (109.6, 67.0) |
| | 393 K | 24 THR (-30.9, 83.5); 128 PHE (98.6, 42.4) |

Table 4.5 shows the outliers in each of the final structures after the simulation along with the original protein obtained from the PDB web database. It can be noticed that few of the residues have been repeatedly shown responsible for causing steric clashes and thus became outliers on the Ramachandran plots obtained for proteins treated at different parameters. The most common residue that has been recorded seven times as an outlier in the eight simulations is 128-Phe. This deviation from the normal orientation of the φ-ψ torsion angles might be due to its structure (Vanga, Singh, & Raghavan, 2015a). Because it is an aromatic compound, there would be a higher steric interference due to the six carbon ring on the side chain. Apart from this, the final conformation also depends on the tendency of the surrounding molecules towards water, possibility of hydrogen bonding in the region, ionic strength and various other parameters (Kainosho & Ajisaka, 1975) 40-Pro and 168-Pro (including 167-Lys and 169-Leu) have been found to be outliers on multiple protein structures. Prolines exhibit unique properties and are always deviated from the standard convention. These residues were found to form 'turns' or 'coils' (along with the pre-proline residues) which was common in various high resolution structures (MacArthur & Thornton, 1991). On the whole, with about 93% of residues in favoured regions and 98% residues in allowed regions, the final structures obtained after the simulations were very stable at the given experimental time scale.

## 4.5 Conclusion

The molecular simulations showed that the STI molecule is quite stable because of its unusual β-sheet structure under the influence of oscillating electric fields in five nanoseconds. It did not produce unusual deviations due to oscillating fields as observed in the case of Ara h 6. Further, lower deviations in RMSD along with Ramachandran plots where majority of the residues are in

favoured and allowed regions showing that the molecule obtained in fact of a stable conformation. The simulation data also supports the original experimental predictions of other researchers about the role of aromatic residues on the functionality of the STI molecule. Predictions that the solvent accessible surface area of core aromatic residues would increase with rise in temperature which was proved right in the simulations conducted on soybean trypsin inhibitor protein with increasing temperature (300 K to 394 K) both with and without oscillating electric field. It was found that the core tryptophan residues in fact increased significantly, but then again decreased when the treatment temperature crossed 373 K in both the cases. The results also show that the solvent accessible area of cysteine residues (20 $Å^2$) increase with an increase in the temperature (300K to 394K), both in the presence and absence of the oscillating electric field. This increase in SASA of Cys residues can directly influence the disulphide bridge stability as it would provide access for external solvents and enzymes to act on the protein. The complete denaturation of proteins may occur at increased time frames (> five nanoseconds). Our study also provides a new path where MD simulations can be used to verify the experimental predictions regarding the stability of secondary structures in proteins and enzymes.

## CHAPTER V

SUMMARY AND CONCLUSION

Trypsin inhibiors are a group of serine protease enzymes, and are of high importance as they are most common and problematic anti-nutrient present in various crops, nuts and vegetables such as soybean, rice bran, chick pea, sesame seeds, potatoes, lentils, varieties of beans, peanuts, wheat, barley, cucumber, corn and pumpkin. They deviate the normal process of digestion, as they hinder the biological function of trypsin and chymotrypsin in the human body. Hence elimination of trypsin inhibitors from food products is one of the major concerns of food processing industries.

The review discusses various processing methods adopted by the food industry and food researchers to increase and improve the nutritional and functional properties of soybeans by reducing the trypsin inhibitor activity. Soybean is one of the legume that accounts for the highest amount of protease inhibitors, about 2-6% of soybean protein. Processing plays an essential role in utilizing raw soybeans in food and feed industries. An optimal process increases soybean protein quality by improving its texture, colour, flavour, functionality, and digestibility and is also, cost effective and energy efficient. These treatments mainly include heating, pH adjustment, hydrolysis and covalent attachment of other constituents. The review also highlights on the novel techniques of molecular dynamic simulation for understanding the structural mechanism of the proteins involved during processing.

The primary objective of this work was to study effects of microwave processing on soymilk protein, at suitable conditions of time and temperature, to increase its *in-vitro* protein digestibility and eliminate trypsin inhibitor activity to safe levels, in comparison to conventional thermal treatment. With the response surface analysis, it was clearly observed that the *in-vitro* protein digestibility of microwave processed soymilk increased with the increase in the processing time.

The observations of slight decrease in IVPD and then gradual increase, can be related to the changes in the conformation of proteins under the influence of oscillating electric field of microwave. The trypsin inhibitor activity (TIA) studies showed that temperature played the most significant role in the reduction of trypsin inhibitors in the microwave processed soymilk samples. Therefore microwave processing of soymilk can be used as a method of processing soymilk with inactivated anti-nutritional factors and increased digestibility.

The secondary objective of the study was to evaluate the applicability of molecular dynamic simulation for studying the effect of thermal and oscillating electromagnetic fields on food proteins. In this thesis, the structural changes of soybean trypsin inhibitor (1AVU) protein were evaluated at different process simulation conditions of time and temperature using GROMACS software, for understanding its behaviour at molecular level. The Soybean Trypsin Inhibitor (STI) protein with unusual β-sheet structure was quite stable under the influence of oscillating electric fields within the time frame five nanoseconds, which has been analysed using Root Mean Square Deviation (RMSD), Solvent Accessible Surface Area (SASA), Ramachandran plot and Secondary Structure Analysis. Further, in order to inactivate this protein, the disulphide bonds have to be cleaved. The solvent accessible surface area of cysteine residues increased with rise in temperature in the simulations conducted with increasing temperature (300 K to 394 K), both with and without oscillating electric field. This could directly influence the disulphide bridge stability. This study has showed that the molecular modeling simulation techniques can be applied to study the proteins, to predict the structural changes of the food proteins that would help engineer high quality food products with superior nutritional output and digestibility.

FUTURE WORK

1. The experimental studies using microwave technique can be conducted at higher temperatures (>100°C) for shorter time to check for the effective inactivation of soybean trypsin inhibitors and increased digestibility.
2. Molecular dynamics (MD) simulations can be conducted at higher electric field intensities like 0.8 - 2 V/nm to observe the possibility of further disruptions to the secondary structure of the proteins.
3. MD studies can be conducted for extended periods of time like 10 ns – 50 ns for more extensive data on the various parameters.
4. Trypsin inhibitors can be quantified in more biological sample proteins for a better understanding of these protease inhibitors.

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APPENDIX

**Figure 1:** Ramachandran plot of 1AVU protein simulated under no electric fields generated using MolProbity (a) 300 K (b) 343 K (c) 373 K (d) 394 K

Figure 1(a)

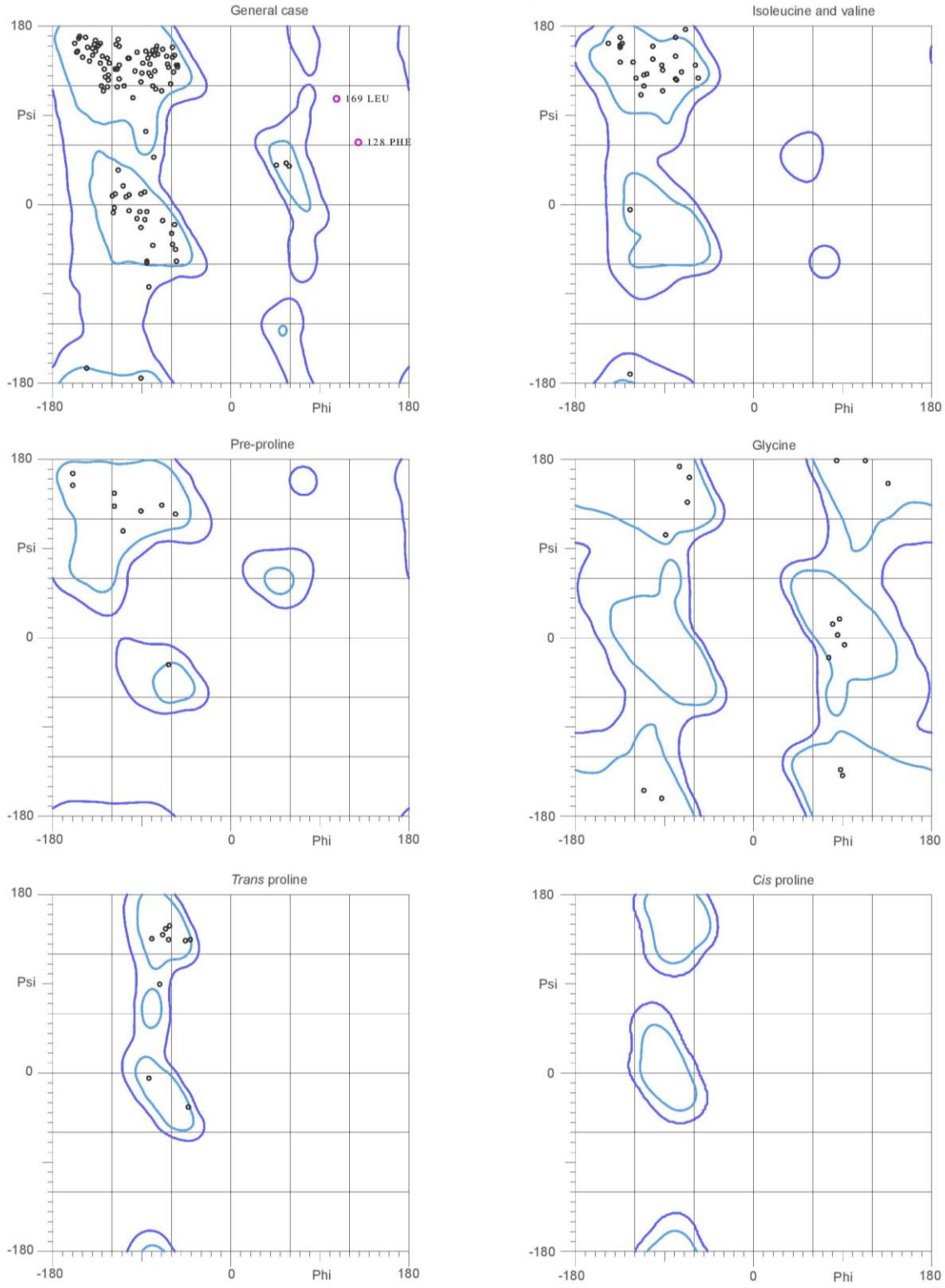

General case
169 LEU
128 PHE
Isoleucine and valine
Pre-proline
Glycine
*Trans* proline
*Cis* proline
Psi
Phi
180
0
-180

Figure 1(b)

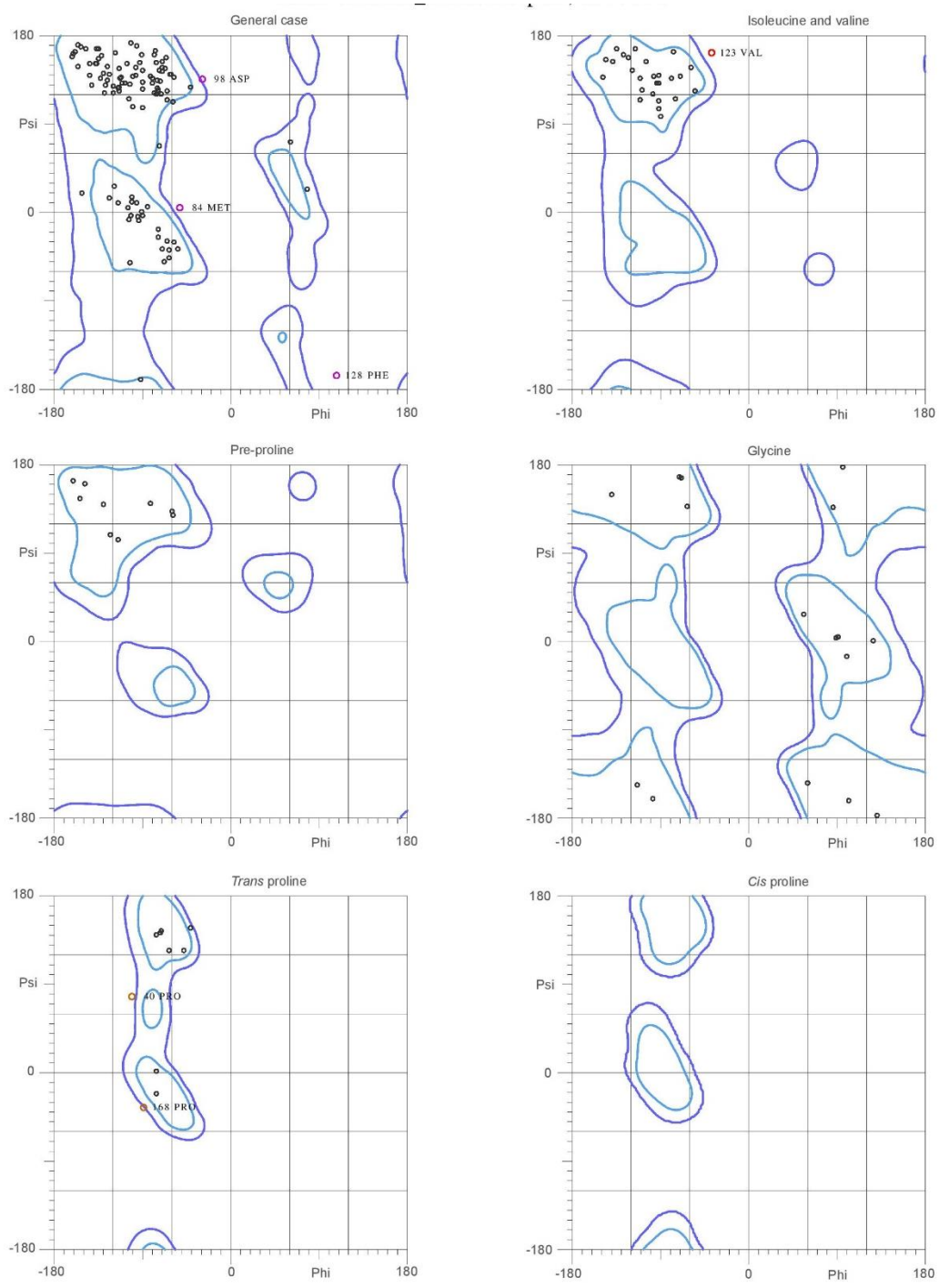

General case
98 ASP
84 MET
128 PHE
Isoleucine and valine
123 VAL
Pre-proline
Glycine
Trans proline
40 PRO
168 PRO
Cis proline
Psi
Phi
180
0
-180

Figure 1(c)

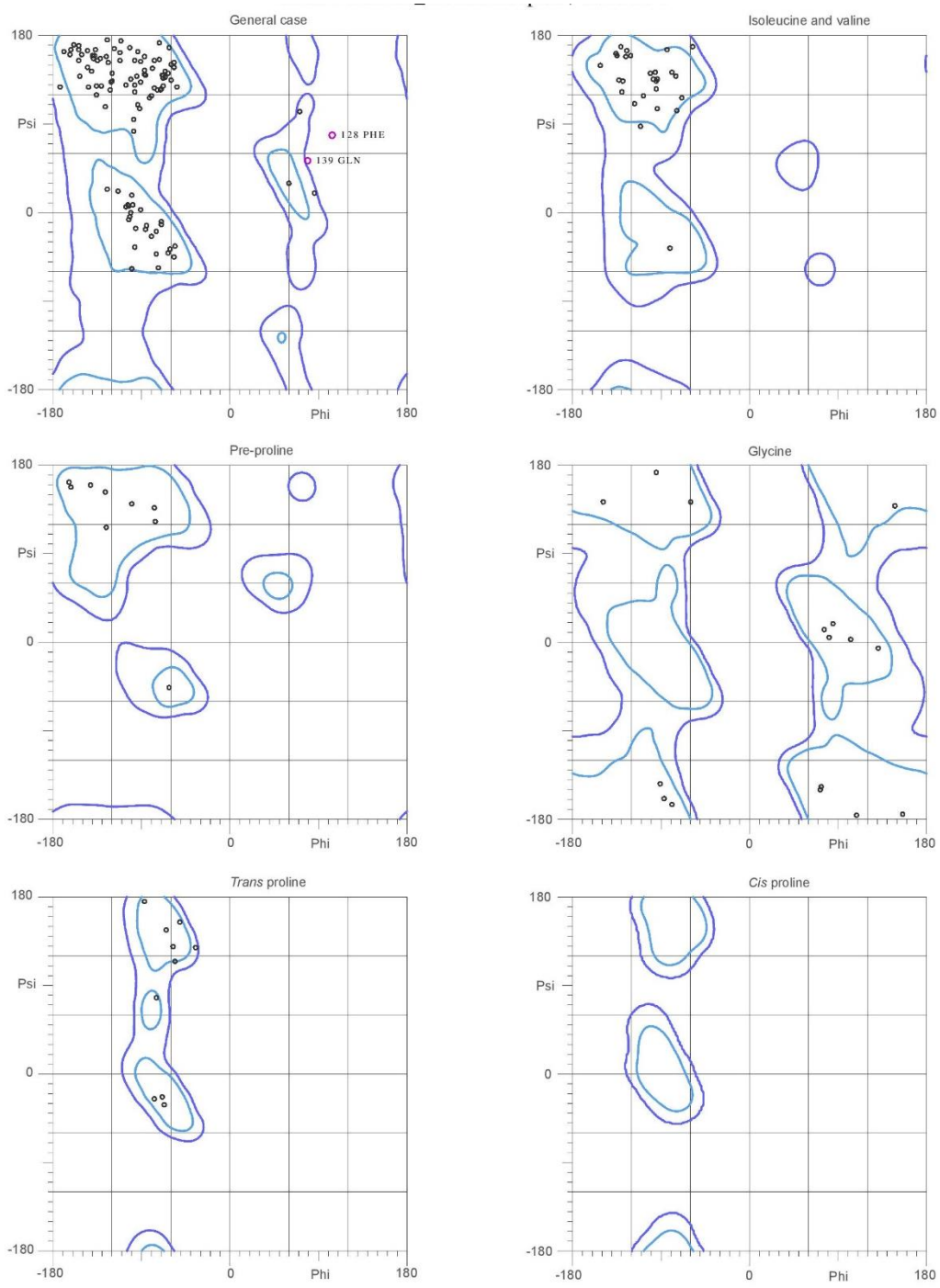

General case
128 PHE
139 GLN
Isoleucine and valine
Pre-proline
Glycine
Trans proline
Cis proline
Psi
Phi
180
0
-180

Figure 1(d)

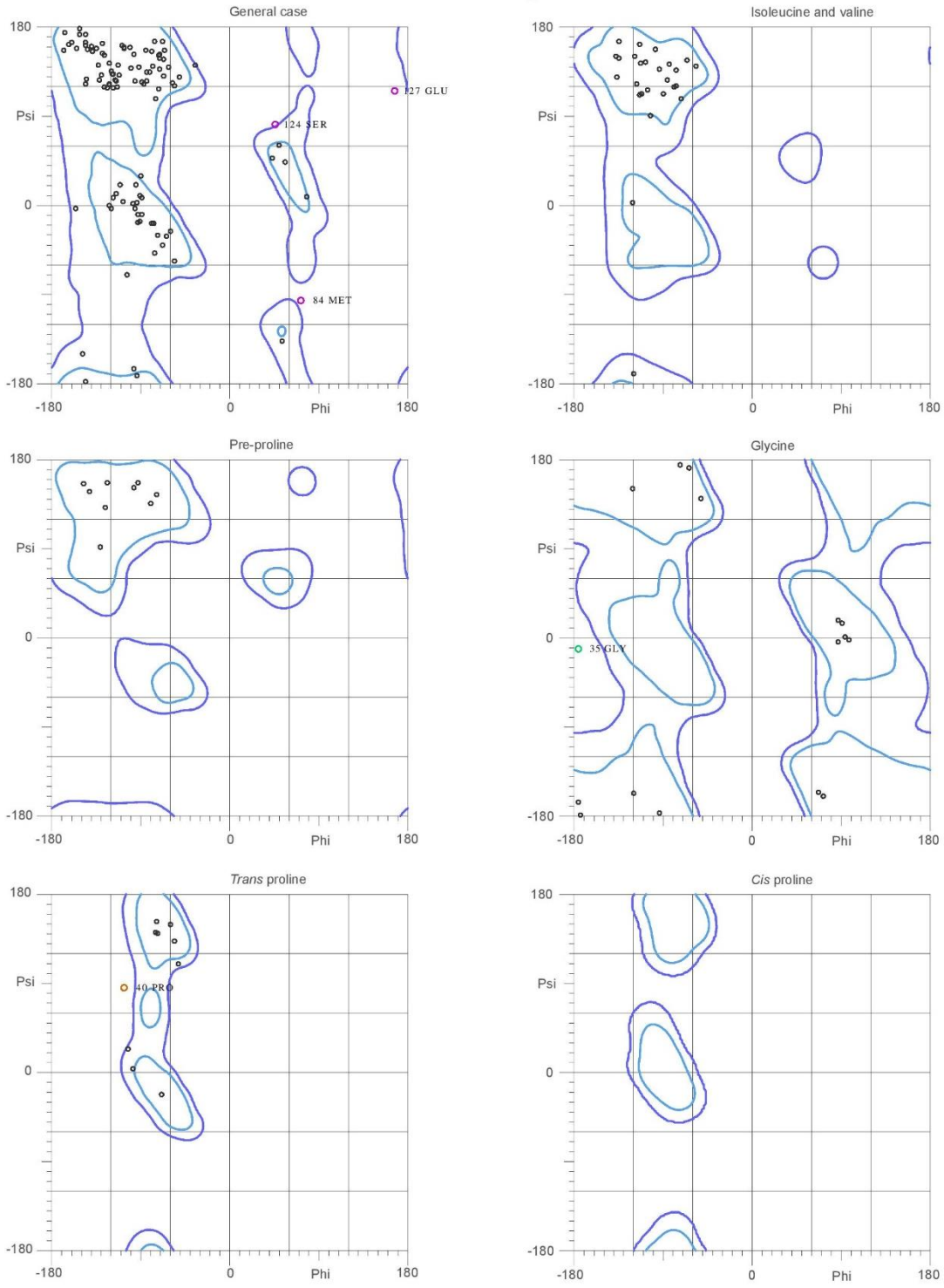

General case
Isoleucine and valine
Pre-proline
Glycine
Trans proline
Cis proline
Psi
Phi
180
0
-180
127 GLU
124 SER
84 MET
35 GLY
40 PRO

**Figure 2:** Ramachandran plot of 1AVU protein simulated under oscillating electric field of 0.5 V/nm and 2.45 GHz generated using MolProbity (a) 300 K (b) 343 K (c) 373 K (d) 394 K

Figure 2(a)

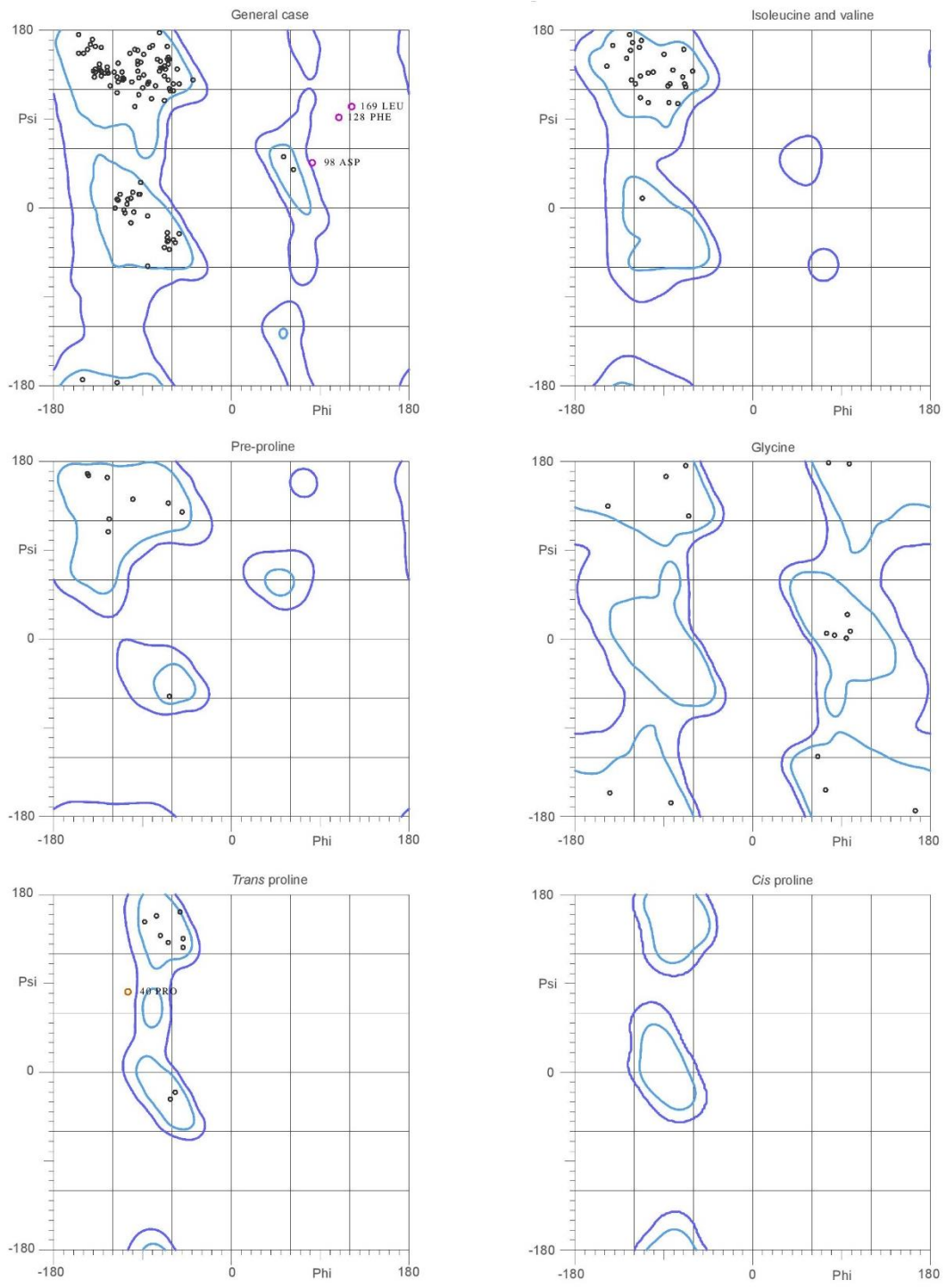

General case
169 LEU
128 PHE
98 ASP
Isoleucine and valine
Pre-proline
Glycine
Trans proline
46 PRO
Cis proline
Psi
Phi
180
0
-180

Figure 2(b)

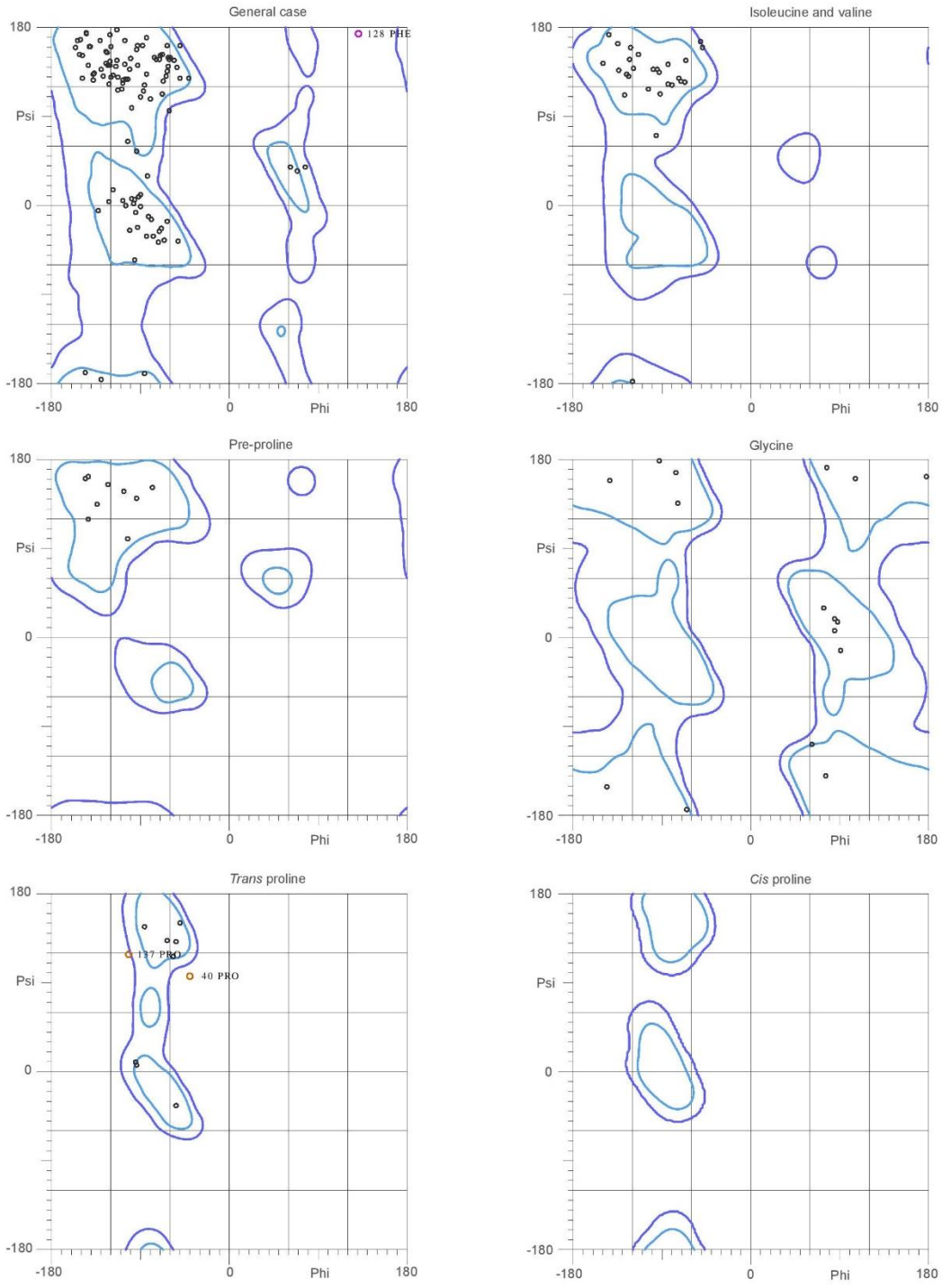

General case
128 PHE
Isoleucine and valine
Pre-proline
Glycine
Trans proline
137 PRO
40 PRO
Cis proline
Psi
Phi
180
0
-180

Figure 2(c)

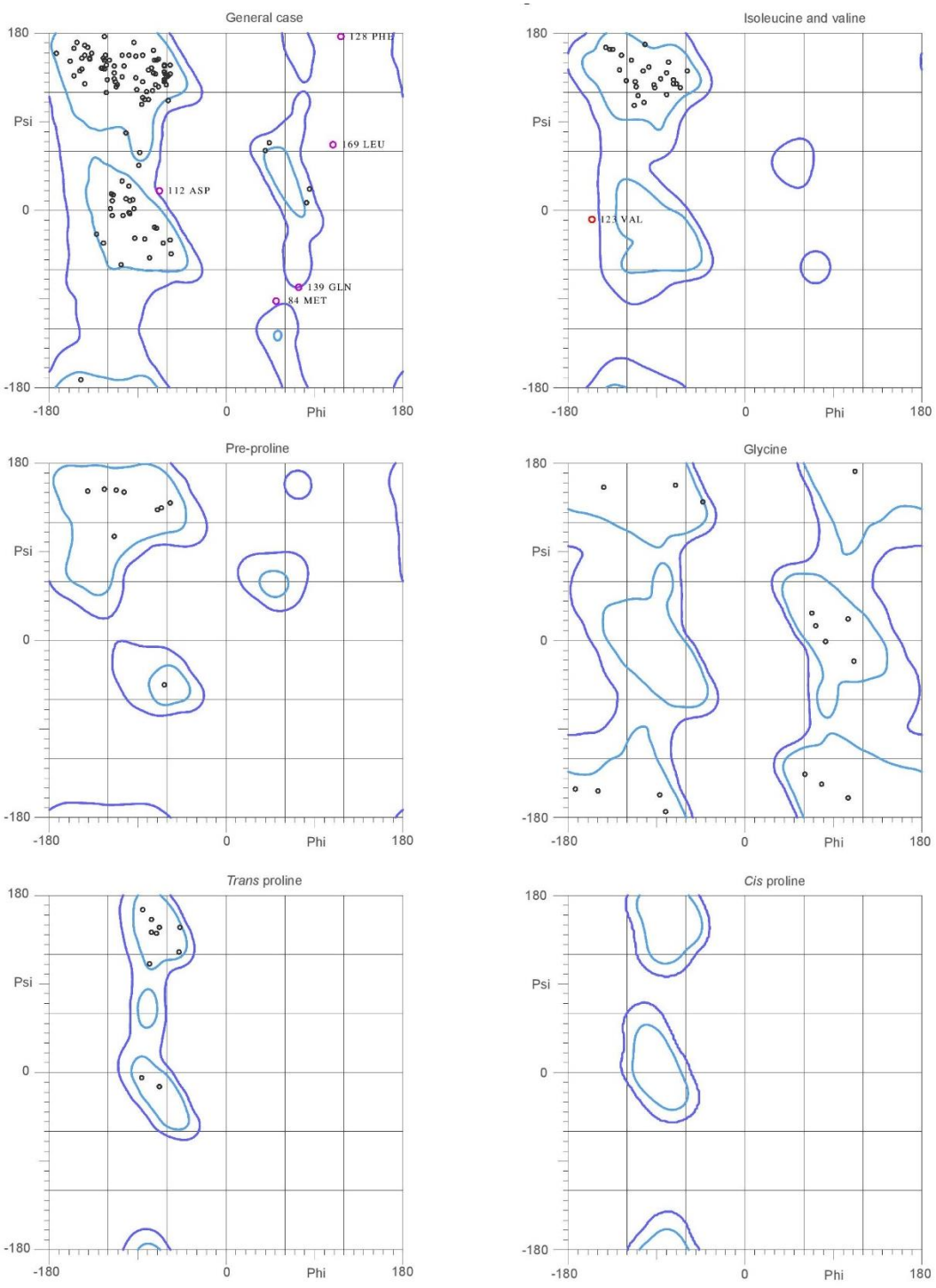
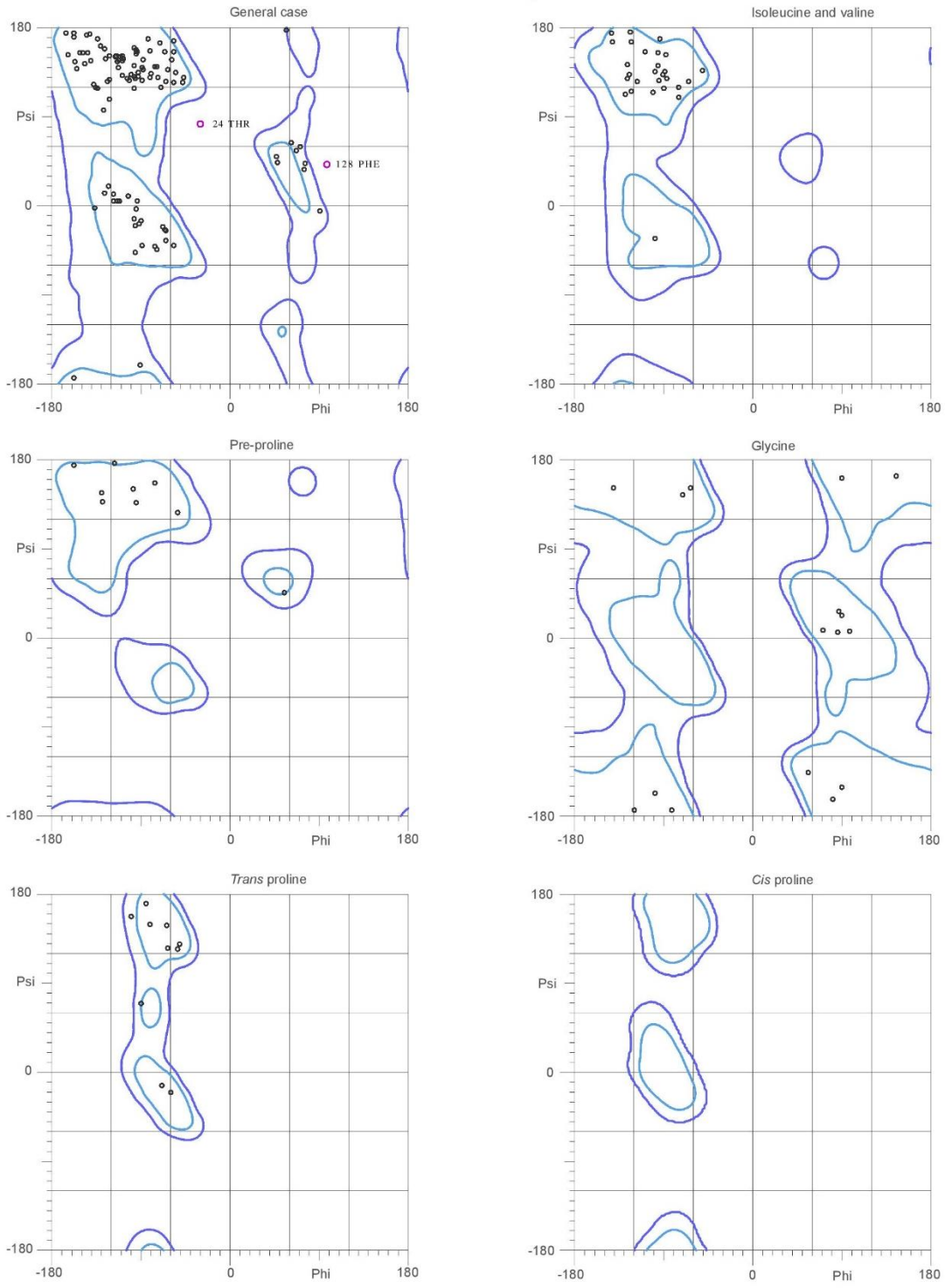

General case
128 PHE
169 LEU
112 ASP
139 GLN
84 MET
Isoleucine and valine
123 VAL
Pre-proline
Glycine
Trans proline
Cis proline
Psi
Phi
180
0
-180

Figure 2(d)


General case
Isoleucine and valine
Pre-proline
Glycine
Trans proline
Cis proline
24 THR
128 PHE
Psi
Phi
180
0
-180