HIGH PRESSURE AND MICROWAVE ASSISTED GENERATION AND PYROLYSIS-GC/MS ANALYSIS OF GLYCATED PROTEINS

By

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment for the requirements for the degree of Master of Science.

August, 2002

Your file Votre référence
ISBN: 0-612-88248-9
Our file Notre référence
ISBN: 0-612-88248-9

DEDICATION

This thesis is dedicated to my mother (Wendy), my father (David) and my sister (Margaret).

ABSTRACT

The extent of denaturation and glycation of lysozyme and BSA with the application of high hydrostatic pressure (HHP) at 400 MPa at 30°C from 8 to 48 hours and focused microwave irradiation at 50°C under varying microwave power and from 10 to 60 minutes was investigated in the presence and absence of D-glucose. The HHP treatment caused 10 to 20% denaturation of lysozyme whereas microwave irradiation caused 20 to 40% denaturation, with more destruction to the lysozyme in the presence of glucose compared to the control. The extent of glycation was also higher with the high pressure samples, causing 60% glycation upon 8 hours of high pressure exposure, but decreasing to around 40% thereafter. Microwave irradiation brought about 40% glycation to the lysozyme samples upon 20 min of irradiation. BSA, on the other hand, was more susceptible to damage by high energy exposures. BSA samples were denatured to a greater extent compared to lysozyme, up to 80% upon the prolonged exposures, but in all treatments, glucose seemed to act as a protectant contrary to the case of lysozyme. The extent of glycation detected was also minimal, ranging from 8 to 20%.

The feasibility of analyzing glycated proteins using pyrolysis-GC/MS was also investigated. Taking advantage of the formation of a diagnostic marker - 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one - upon pyrolysis of glycated proteins, the intensity of this peak was used to correlate the extent of glycation. The intensity of this peak in the pyrograms of glycated lysozymes was found to increase linearly with increasing incubation times and subsequently with the sugar loads of the glycated lysozyme. In addition, using the pyrograms as unique fingerprints, the extent of structural changes between modified and unmodified proteins were also assessed.

RÉSUMÉ

L'étendue de la dénaturation et de la glycosylation du lysozyme et du BSA sous l'effet d'une pression hydrostatique élevée (High Hydrostatic Pressure (HHP)) de 400 MPa à 30°C pour des temps variant entre 8 et 48 heures, de même que l'irradiation sous champ micro-ondes focusé à 50°C sous puissance variable et à des temps variant entre 10 et 60 minutes, fut examinée avec et sans D-glucose. Le traitement HHP donne un taux de dénaturation du lysozyme de 10 à 20%, tandis que l'irradiation micro-ondes donne un taux de dénaturation de 20 à 40%. La destruction du lysozyme est aussi plus grande en présence de glucose lorsque l'on compare à l'échantillon de contrôle. L'étendue de la glycosylation est également plus grande avec les échantillons soumis au traitement de forte pression, donnant une glycosylation de 60% après 8 heures d'exposition au traitement, mais diminuant à environ 40% par la suite. L'irradiation sous champ micro-ondes a donné environ 40% de glycosylation pour les échantillons de lysozyme après 20 minutes d'irradiation.

D'autre part, le BSA s'est avéré plus susceptible aux dommages lors de son exposition au traitement sous forte énergie. Les échantillons de BSA se sont dénaturés d'avantage que ceux du lysozyme, soit jusqu'à 80% lors de longues périodes d'expositions, mais dans tous les traitements, le glucose a semblé agir comme un agent protecteur, contrairement au cas du lysozyme. L'étendue détectée de la glycosylation fut également minimale, variant entre 8 et 20%.

Une étude de faisabilité pour l'analyse des protéines glycolysées par pyrolyse en GC/MS fut également examinée. Prenant avantage de la formation d'un marqueur diagnostique, le 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, sous pyrolyse de protéines glycolysées, l'intensité du pic fut utilisée pour corréler l'étendue de la glycolysation. On a trouvé que l'intensité de ce pic dans le pyrogramme des lysozymes glycolysées augmentait de façon linéaire avec les temps d'incubations plus longs et conséquemment avec le nombre de sucres présents dans les lysozymes glycolysés. De plus, l'utilisation des pyrogrammes comme empreintes uniques, a permis d'évaluer l'étendue des changements structuraux entre les protéines modifiées et les protéines non-modifiées.

ACKNOWLEDGEMENTS

Believe it or not, this is the section that I have been thinking of what to put down since I started my master studies. I really want to express my thanks and deep gratitude to everyone who provided advice, supervision, help, encouragement and support to allow me to get to this point. It has been a team effort in completion of this work.

First of all, I would like to express my deep gratitude to my supervisor, Dr. V.A. Yaylayan. His advice and guidance were crucial to my thesis. I sincerely appreciate the time and thoughts given by him, in which, many of his invaluable advice, ideas and comments have been included in this thesis. His help was always there when needed. And most of all, his patience and encouragement made my life way easier and more pleasant throughout my graduate study.

Dr. H. Ramaswamy, as my co-supervisor, for allowing me to have access to the high pressure equipment. He also gave me the chance to be the teaching assistant in two of his courses, and provided helpful guidelines in how to make the class more dynamic, which I find it very valuable in training myself to be more organized and expressive. In addition, thanks to the team of graduate students in the pilot plant as well, who took their time to assist me to operate the high pressure equipment.

Dr. J. I. Boye, who participated in meetings and gave valuable comments on the results of my experiments. But I should have thanked her two years ago when I did a summer project at St-Hyacinthe. What she taught me at that time made me very well prepared when I started my study, and as well, when I come out to work in the future.

Special thanks go to Dr. F. K. Yeboah who provided constructive suggestions and comments on the results of my experiments, that were decisive.

Throughout these two years in achieving my master degree, the continuous support and encouragement from my family made my dreams come true. Distance-wise is far, but mentally close with my mom and other family members who are in Hong

Kong, and to my sister, aunty Mimy and uncle, and other relatives who are in Montreal, thank you for supporting and sustaining me and thanks dad, I miss you still.

Time in the lab would be very much different without Andre, Luke and Eva. The fun talks and laughs created a very joyful and comfortable atmosphere in the lab. Special thanks to Andre for teaching me how to do pyrolysis properly. Thanks to all of them.

Thanks to Dr. I. Alli and Dr. B.K. Simpson who took their time in counseling and directing me whenever I needed help.

I was much stronger throughout these years because of the soothing talks and casual chats with Vivian and Mandy. You have been true friends, thank you so much.

Thanks to FCAR for awarding me the graduate scholarship, and to NSERC strategic fund for funding this research.

Last but not least, thanks to Barbara, Lise, together with all other professors and staffs in the food science department, who are always helpful.

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1 INTRODUCTION

The Maillard reaction, which involves the interaction of amino acids or proteins with reducing sugars, has been the subject of investigation by food scientists and by the food industry, due to its effect on over-all quality attributes of processed food. This reaction which is well known to contribute to deterioration of food quality during processing and storage (Reynold, 1965; Dworschak, 1980), at the same time, is also known to impart desirable flavor and color, as well as texture to some processed foods. This dual role of Maillard reaction in food processing makes it necessary to study the means of its control.

Thermal treatment has always been used as a means of processing food to generate desirable flavors and aromas. However, heat processing generally causes degradation of thermally sensitive nutrients, vitamins, colors, flavors and texture (Khamrui and Rajorhia, 2000). Novel technologies with minimal processing of food, such as high hydrostatic pressure and microwave irradiation, can overcome some of these undesirable side effects of high temperature processing.

Application of high hydrostatic pressure to food systems has been used for decades as a means to preserve food, and it is only in the last 15 years that scientists started to investigate the effects of high pressure on chemical reactions, specifically Maillard type reactions. With an increase in the activation volume due to the formation of volatiles and other small molecules that are important for flavor production, the rate of the final stage of reaction is retarded or suppressed with the application of high pressure

(Tamaoka *et al.*, 1991; Issacs and Coulson, 1996; Bristow and Issacs, 1999; Schwarzenbolz *et al.*, 2000); while the rate of the formation of the initial condensation products (Amadori products) is accelerated. This important property can be exploited to generate glycated proteins to modify the functional properties of different food related proteins.

Microwave ovens on the other hand, have always been used as a means of reheating foods by the consumer (Schiffmann, 1994). Due to its property of high degree of penetration and rapid heat transfer, food manufacturers have been trying to exploit ways to process foods such as tempering of frozen fish or meat and drying of fruits and vegetables (Ramaswamy and Van de Voort, 1990; International Review, 1997). Microwave energy can also be used to carry out chemical reactions such as Maillard reaction (Yaylayan, 1996). Maillard reaction between tofu and glucose was observed with a considerable weigh loss and decrease in solubility of tofu upon microwaving (Kaye *et al.*, 2001).

In foods, proteins play important functional role in dictating the overall perception of the food products. Some of the functional properties include solubility, viscosity, water binding capacity, gelation ability, elasticity, etc to name but a few. Glycated proteins, with controlled attachment of glucose to the proteins, can lead to a decrease or an increase in solubility (Kato *et al.*, 1978; Handa and Kuroda, 1999) or enhancement of emulsifying properties, with a decline in gel strength upon the

occurrence of Maillard reaction (Miyaguchi *et al.*, 1999). Therefore, depending on the type of product to be manufactured, the extent of glycation should be controlled.

This thesis will investigate the effect of high hydrostatic pressure and microwave irradiation on glycation of two common food proteins, lysozyme and BSA, using Lowry test (Lowry, 1951) and fluorescamine assay (Yaylayan *et al.*, 1992) to determine the amount of soluble proteins and the extent of glycation. In addition, a fast and convenient method based on Pyrolysis-GC/MS will be developed to analyze glycated proteins.

2 LITERATURE REVIEW

2.1 INTRODUCTION

Maillard reaction, or non-enzymatic browning, is a chemical interaction that occurs widely in food and biological systems. It refers to the reactions initiated by the interaction between an amino group and an α-hydroxycarbonyl moiety of a reducing sugar (Yaylayan, 1997). In food systems, the primary source of free amino compounds comes from the amino acids in the proteins; while reducing sugars are the primary source that supplies the carbonyl groups. This reaction governs the formation of color and flavor in foods, as well as the properties of the proteins involved. However, the Maillard reaction, also known as glycation when proteins are involved, is not just a single reaction, but a cascade of complex reactions depending on the corresponding precursors and the reaction conditions (Weenen *et al.*, 1997). Since the discovery of browning between amino-carbonyl compounds by Maillard in 1911, the chemistry of the Maillard reaction has been extensively studied, as well as the different aspects in which Maillard reaction plays a role, specifically in food and physiological systems. A brief summary of glycation and the chemistry of Maillard reaction will be presented in this review.

2.2 THE MAILLARD REACTION

2.2.1 CHEMICAL PATHWAYS

The complexity of the Maillard reaction lies in the fact that the reaction is difficult to stop once it is initiated. It is accompanied with the formation of a multitude of compounds in minute amounts taking place by side reactions and obscure pathways. Maillard reaction can occur even at moderate room temperatures, and the rate of reaction

is accelerated with increasing temperatures. The chemical pathways can broadly be divided into the early, intermediate and the final stages (Hodge, 1953). In the early stage of the Maillard reaction, the carbonyl group undergoes condensation with the amino group with the loss of a water molecule, forming the Schiff base, glycosylamine (Figure 1.1). This compound being unstable, will then undergo subsequent isomerizations to a more stable form. Amadori compound is formed when the amine rearranges with aldose to an amino ketose, while Heyns product is formed when the amine rearranges with ketose to form an amino aldose (Ledl, 1990). Up to this point, reactions are reversible in aqueous solutions, since the glycosylamine can be hydrolyzed into its parent compounds (Namiki, 1988). However, the pH of the system changes as the reactions proceed with the formation of different products. Depending on the pH of the system, different reaction pathways will take precedence in the advanced and final stages of the Maillard reaction, leading to the formation of nitrogenous polymers and co-polymers, known as melanoidins (Figure 1.2). With an alkaline environment having a pH greater than 7, chain fragmentation of the Schiff base and the Amadori compound will occur, leading to the formation of 2- and 3-carbon fragments which will undergo further irreversible reactions to form the brown pigments. With a more acidic or a neutral environment having a pH less than 7, deoxyosones will be formed by eliminating the original amine from the Amadori compound. The deoxyosones are very reactive intermediates and will undergo cyclization or dehydration to form higher molecular weight compounds, generating flavor and color (Rizzi, 1994).

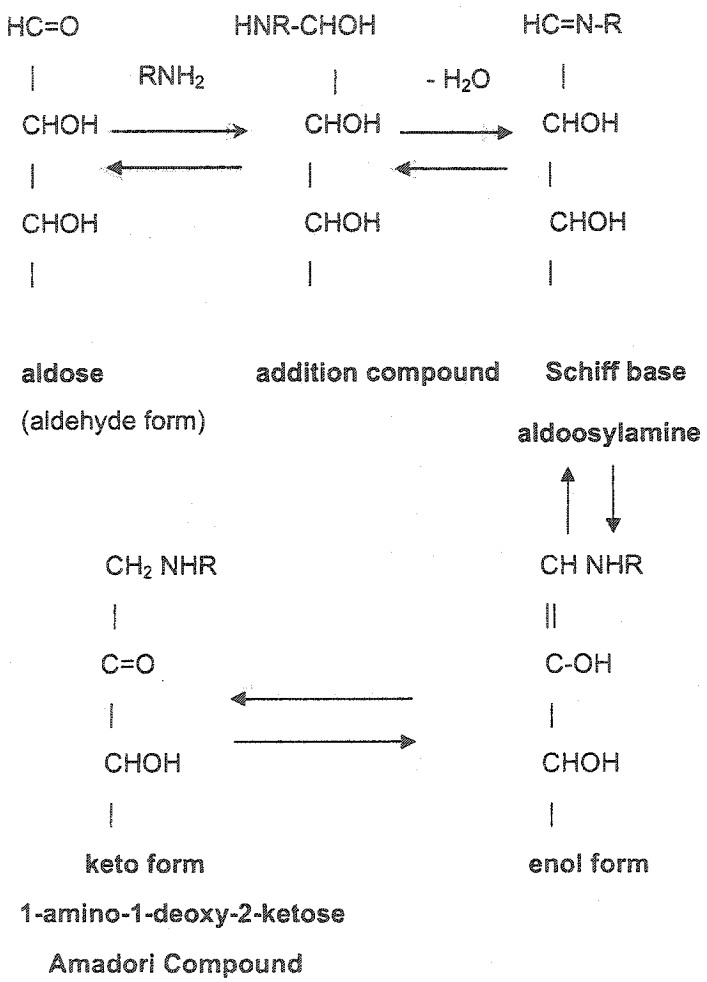


Figure 2.1 Early stage of Maillard reaction between a carbonyl group and an amino group and formation of Amadori compound, showing partial structures.

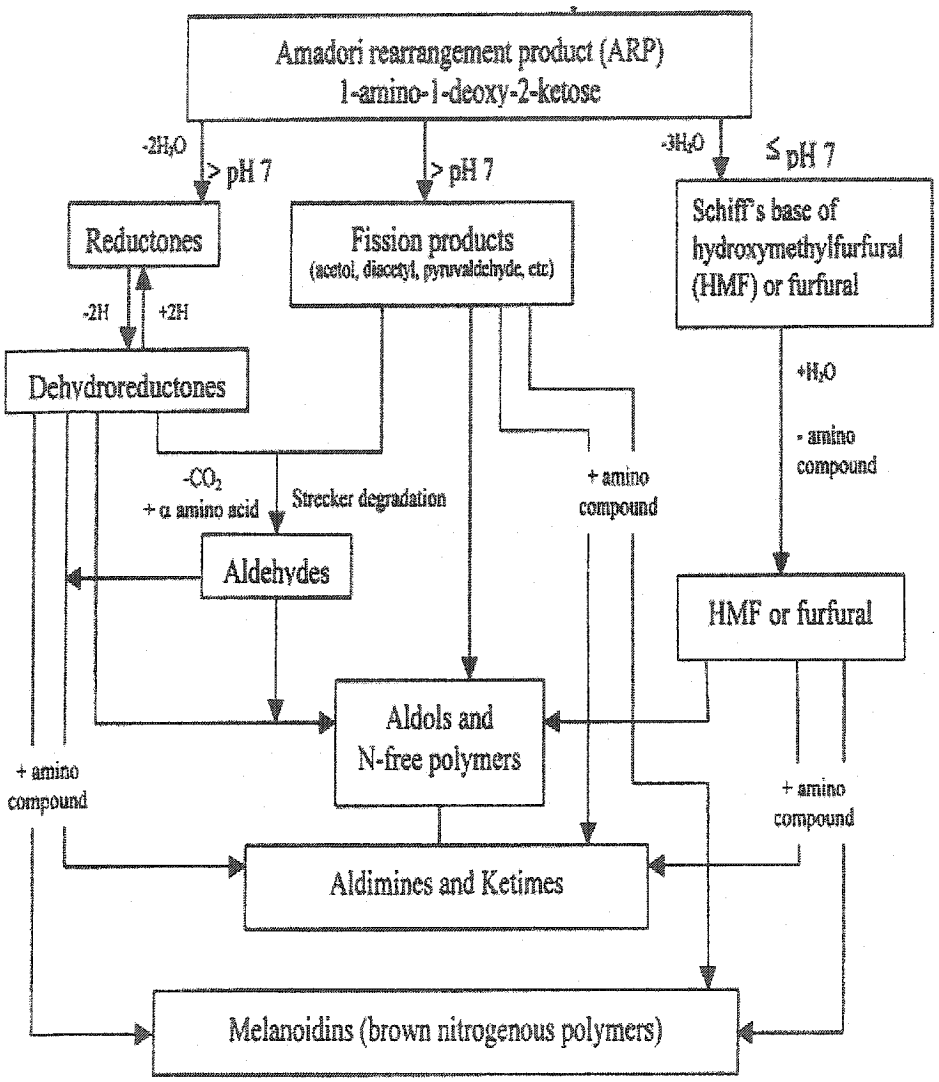


Figure 2.2 Reaction pathways of the advanced and final stages of Maillard reaction adapted from Hodge (1953).

2.2.2 SIGNIFICANCE OF MAILLARD REACTION/GLYCATION IN FOODS

Proteins dictate the corresponding nutritional quality and the functional properties in foods with the type and amount of amino acids present and the corresponding sequence of the amino acids which form the structure of the proteins. Maillard reaction is a reaction that can occur mainly between the γ-lysyl groups of the proteins and the aldehyde groups of reducing sugars, and it occurs even under the mildest conditions. Therefore, with the onset of the reaction, availability of lysine will be significantly reduced (Hurrell, 1990). Proteins that have reducing sugars attached to them are termed modified proteins, and they exhibit different functional properties as compared to the original unmodified proteins. As the reaction proceeds to the advanced and final stages, free lysine could be destroyed, as well as other essential amino acids such as histidine, arginine, tryptophan, cysteine etc, in which they will undergo further reactions with premelanoidins. Thiamine, a vitamin, has an amino group which can also take part in Maillard reaction if present in the food, and can possibly have its bioavailability reduced.

Despite the effects on the nutritional quality of the foods, Maillard reaction is an important chemical reaction to mankind, because it is responsible for generating the flavor and color. With the modifications of proteins, their functional properties can also be changed. Different proteins with different glycation conditions produce different functional properties. Soy protein isolates, for example, when glycated for 3 days showed improved solubility, emulsifying stability and foam expansion, while showing slight decrease in the fat binding and water holding capacity (Boye *et al.*, 2002). Solubility of the bovine serum albumin (BSA)-glucose mixture and the ovalbumin-glucose complex in

their dry state decreased as the incubation period lengthened (Watanabe and Sato, 1980, Yeboah *et al.*, 1999); emulsifying properties, gelling and foaming properties of the ovalbumin-dextran complex were improved as Maillard reaction proceeded (Nakai, 2000). Functional properties of the proteins in food therefore play a significant role in the perception of the food products. Controlled glycation can therefore generate glycated proteins which can be suitable for producing food products with different sensory and functional properties.

Flavor, which refers to a combination of smell and taste, is the other area that governs the overall perception of food products, and is the most important factor of all. Since the discovery of fire by mankind, human beings used thermal energy to cook food to acquire the desired flavor, texture and color. Application of heat to bread-baking, browning of meats and others at the suitable temperatures and for the right duration of time can generate desirable flavors; while on the other hand, over-processing the product can generate unacceptable flavors. The aroma compounds are the smell chemicals that are generated from the Maillard reaction, and are low molecular weight compounds that are usually volatile. They could be formed by first producing lower molecular weight compounds such as pyruvaldehyde from retro aldol reactions of deoxyosones obtained from the initial stage of Maillard reaction. They will then react with the amino acids in the systems to form flavor volatiles (Rizzi, 1994). Other pathways in generating aroma compounds include cyclization or dehydration of deoxyosones. Compounds such as furanones could be generated, which have a distinct smell by itself, but they could also undergo further reactions to generate different flavor, such as reacting with the sulfur in

the system (if present) and generate a wide range of aromas. Because of the numerous possible pathways that could generate different aromas and taste compounds, Maillard reaction is very complex and complicated. Since this review is on the glycation of food proteins, this section will mainly deal with the formation of Amadori compounds when the proteins and sugars are processed using novel technologies.

2.3 FOOD PROCESSING AND NOVEL TECHNOLOGIES

2.3.1 INTRODUCTION

Cooking is a form of thermal processing that is used to add value to the raw materials in order to produce desirable flavors, aromas and texture, and to transform the raw materials to an edible and safe-to-consume form. The food industry utilizes similar principle to commercially produce food products for consumption in a ready-to-eat form like milk or foods with a longer shelf life like canned foods. There has been increasing interest by the food industry to investigate alternate lower-cost technologies to process foods with minimal damage to the food components.

2.3.2 HIGH HYDROSTATIC PRESSURE (HHP) AS A PROCESS

The use of high hydrostatic pressure (HHP) on foods has been around for over a century. Food industry utilizes the ability of high pressure to inactivate micro-organisms to process food to be safe for consumption and have extended shelf life. A typical high pressure system consists of a high pressure chamber with closure in which samples or packages are loaded; a pressure generating system to generate pressure either by direct or indirect compression; and a temperature control device to monitor the temperature of the

pressure chamber either in the form of sensors for large-scale production or thermocouples for laboratory-scale equipment (Ting *et al.*, 2002). Physical-chemical properties of water such as phase transition and density can be modified upon the application of pressure, and can therefore induce protein denaturation and modification of biopolymers in food affecting properties such as gelling, susceptibility to enzymatic degradation, etc. (Knorr, 1994). In addition, losses in nutrients and quality of the products with ultra high pressure can be minimized as compared to thermal processing because the process could be carried out at ambient temperatures, depending on the types of micro-organisms to be destroyed or inactivated. Vegetative cells and yeasts are pressure-sensitive, and they can be destroyed at ambient temperatures with above 100 MPa of pressure (Hoover *et al.*, 1989); bacterial spores, on the other hand, are more resistant to pressure and requires about 600 MPa of pressure at elevated temperatures between 45-60°C (Hayashi, 1992). It must be noted that with such high pressure, food products will not be deformed and will retain their shape after being processed, since pressure is being applied uniformly from all directions on the products. In other words, the operation of high pressure is independent of size and geometry of the product to be pressurized.

Although the capital cost for installing the high pressure equipment is usually high (Meyer *et al.*, 2000), nonetheless, because of its short process times and high quality of foods generated, the use of HHP as a process which has been known for around 100 years, starts to attract the attention of food manufacturers to process food in the last decade, especially those in Japan which demand high quality foods.

2.3.3 MICROWAVE IRRADIATION AS A PROCESS

Microwave energy is the electromagnetic radiation that lies between infrared irradiation and radio waves and has a frequency range between 300 MHz to 300 GHz (Jauchem, 1998). It was discovered Dr. P. Spencer in 1946 while testing a vacuum tube called magnetron (Gallawa, 2002). Microwave energy can be converted into heat by either dipole rotation or ionic conduction. In other words, the presence of either dipolar or ionic molecules can interact with the electric field and generate heat (Yaylayan, 1996). Because microwave irradiation has relatively long wavelengths, it has a high degree of penetration and can therefore rapidly transfer heat throughout the molecules to be irradiated. Hence, microwave processing can save more time in processing as compared to thermal processing.

Invention of the domestic microwave ovens did not gain popularity for use at homes until the last two decades when more women entering the work force, had increased demand for a more convenient tool to reheat food rapidly (Ramaswamy and Van de Voort, 1990). The food industrial sector also took this opportunity to investigate the potential of exploiting microwave irradiation as being an economical means of processing foods because of its rapid heating characteristics. Drying, thawing and thermal processing are some of the common microwave applications in the food industry today (International Review, 1997; Ramaswamy and Van de Voort, 1990). Microwave-dried tarhana (a wheat flour-yogurt mixture) samples exhibited higher overall sensory rating and color acceptability as compared to freeze-dried and tunnel-dried samples (Hayta *et al.*, 2002). Thawing of model frozen foods using microwave also showed a

seven-fold reduction in time as compared to convective thawing at ambient temperature (Virtanen *et al.*, 1997). Industrially, microwave tempering for converting hard-frozen foodstuffs into a workable, usable and tempered form which are not necessarily thawed completely is necessary for frozen food industry for further food processing, in which, the tempered foodstuffs did not show any quality loss (Decareau, 1985). Focused microwave heaters are also used to pasteurize and process foods such as dairy products and nutritional products like protein beverages. A focused microwave system differs from the domestic microwave oven in that the microwave irradiation is being delivered to the object to be irradiated directly in a beam, instead of allowing the irradiation beams to bounce back and forth on the walls of the microwave oven before hitting the sample. Microwaves in this case are being focused throughout the volume of the product, allowing for more uniform and instantaneous heating, with minimal loss in flavor and color (Clark, 2002).

2.3.4 PROS AND CONS OF MINIMAL PROCESSING

Minimal processing offers the advantage of keeping the food safe and extending its shelf life. Product quality can as well be improved in retaining more of the thermally-sensitive nutrients, colors, and flavors as compared to regular thermal processing. However, the lack of Maillard reaction in producing the desired colors, flavors and textures to the food product as compared to thermal processing poses a major disadvantage to the consumer acceptance.

2.4 MAILLARD REACTION AND NOVEL TECHNOLOGIES

2.4.1 MAILLARD REACTION AND HHP

Other than being used for destroying micro-organisms, HHP can influence chemical reactions depending on the changes in the activation volumes that occur. A kinetic study by Tamaoka *et al.* (1991) concerning the effect of chemical reaction under HHP showed that when the activation volume decreases with the production of smaller number of molecules having less activation volume (3.9-8.9 mL/mmol), high pressure favors the Maillard reaction. On the other hand, when the reaction will generate more molecules, no matter whether they are small in size or not, they will have a greater activation volume (12.8-27.0 mL/mmol) and will not be favored. Therefore, in the early stage of Maillard reaction, formation of the Amadori compound from the amino acids or proteins with the carbonyl groups of reducing sugars (resulting in only one ketosamine compound) will be favored by high pressure, while the later stage of browning that forms numerous volatile compounds and color will not. Okazaki *et al.* (2001) used white sauce made from milk, flour and butter as a model food to study the effect of HHP on browning. It showed that the white sauce that was heated without HHP treatment browned more significantly than the white sauce being subjected to pressurization with heating to 115°C for 30 minutes. This further confirms that HHP suppresses the Amadori product from proceeding to the browning reaction which happens in the advanced and final stages of the Maillard reaction. Issacs and Coulson (1996) also suggested the accumulation of Amadori product under HHP due to the retardation in forming advanced Maillard reaction products that come from the degradation of the Amadori rearrangement product (ARP).

2.4.2 MAILLARD REACTION AND MICROWAVE IRRADIATION

Although microwave irradiation can serve a wide variety of uses in the food industry, it is rarely used to cook food because of its incapability to generate Maillard flavors. The presence of the ionic or dipolar molecules in food in which microwave irradiation has an effect are usually in the form of water within the food matrix. In other words, when the food is being microwaved, the highest temperature is not at the surface, resulting in the incapability to form a crust, leading to reduced flavor and less color formation (Lingnert, 1990). This also explains why the general public mainly use microwave oven to reheat leftovers of food or defrost frozen meat, and rarely use it for cooking to generate the desired flavors for consumption (1997 Consumer Survey Summary).

To overcome the lack of flavor and color development of microwave food products, different strategies have been attempted. Some aimed at the modification of the cooking environment by using absorbing susceptor sheets or special microwave browning pans (Van Eijk, 1994); some combined microwave cooking with thermal heat (Eke, 1997); some added commercial flavorings or special coatings to food product prior to microwaving to produce the desired aromas and color (Reineccius and Whorton, 1990). Food scientists also tried to investigate the possibility of generating desired flavor compounds with the microwave technology. A reaction system of proline and glucose was used to compare the effects of thermal and microwave mediated reactions on Maillard reaction (Parliament, 1993). The GC/MS analysis of the samples revealed that the volatiles produced from the thermally treated systems were generally the same qualitatively as that from the microwave treated systems, but the microwave samples had

larger quantities of carbohydrate decomposition products while the thermally treated samples had larger quantities of N-heterocyclic compounds. Yaylayan *et al.* (1994) also initiated Maillard reaction under microwave conditions by having sufficient concentrations of the reactive Maillard precursors in the model systems consisting of different amino acids with glucose. However, no studies have been reported on protein glycation by microwave irradiation.

2.5 METHODS OF DETECTING GLYCATED PROTEINS

The detection of glycated proteins formed from Maillard reaction is made possible by a number of chemical and analytical methods. Chemical methods assay the sugar-bound protein or protein-bound sugar via chemical reactions, with the detection of the product formed either colorimetrically or fluorometrically. In the thiobarbituric acid (TBA) method, the glycated protein is heated in oxalic acid to release the sugar that was previously attached. The sugar-free protein is then removed by acid precipitation, while the sugar that remained is converted into 5-hydroxymethylfurfural (HMF). The condensation product of HMF with TBA can be detected by spectroscopy or analytical methods such as high pressure liquid chromatography (HPLC) (Fluckiger and Gallop, 1984; Furth, 1988). Borohydride reduction works by reducing the C=O and C=N of ARP with sodium borohydride under alkaline conditions (Mendel, 1996; David *et al.*, 1998), followed by subsequent methods in quantifying the amount of glycated proteins that are present. Siciliano *et al.* (2000) digested the reduced protein with trypsin and analyzed the resulting mixture with matrix assisted laser desorption ionization (MALDI) mass spectrometry. The fructosamine method, or nitroblue tetrazolium (NBT) method, is a

common method that is used to estimate the amount of glycated serum protein in diabetic patients, in which, the NBT dye reduces the glycated protein to form a blue product that can be detected at 540 nm (Furth, 1988). Other color tests for the presence of ARP such as ferricyanide test in which glucose reduces ferricyanide to ferrocyanide that is blue in color (Borsook *et al.*, 1955). All these tests are simple, but they are very susceptible to interference by other compounds in the system such as the presence of free sugars. Removal of the free sugars by dialysis prior to testing could be performed, but this will increase the overall time for the analysis.

Analytical assays based on separation of the glycated species from the unglycated species in the mixture could be done by using different types of chromatography, in which, the protein itself is being assayed, and hence eliminating the need to separate the protein from the unreacted sugars prior to analysis. However, many of these separation-based assays are highly selective. For example, phenylboronate affinity chromatography can separate glycated proteins efficiently, provided that the sugars that are being attached to the ARP are not phosphorylated since they will be weakly adsorbed to boronate resins, leading to underestimation of glycation.

The ARP can alternatively be transformed to compounds that fluoresce and can be measured as such. The furosine method measures the amount of furosine that was formed upon acid hydrolysis of ARP. The furosine formed is a specific product of lysine ARP, and accompanying its formation is pyridosine which is also formed in minor amounts (Furth, 1988; Marconi *et al.*, 2002), both are separated and quantified by HPLC at 280 nm. A non-separation fluorescence quenching assay measures the fluorescence

generated when a fluorescein-boronic acid derivative is quenched with the glycated protein at an excitation wavelength of 450 nm and an emission wavelength of 525 nm (Blincko *et al.*, 2000). Fluorescamine assay is another method that can be used to detect the glycated proteins. Glycated proteins are brought to alkaline conditions with borate buffer (pH 8.5), fluorescamine is added to the solution and the fluorescence is measured at an excitation wavelength of 390 nm and an emission wavelength of 475 nm (Yaylayan *et al.*, 1992). Fluorescamine reacts with ε-amino groups of lysine to generate fluorescence, while reducing sugars will not react. This assay measures the relative extent of glycation with respect to the blank that contains no protein. In addition, prepared glycated proteins can be assayed without the removal of sugars, because the assays are targeted on the protein or the ARP, and not the sugars.

In this study, the effect of HHP and focused microwave irradiation on glycation of lysozyme and BSA will be investigated. The extent of glycation and protein denaturation that occurred upon subjecting the models to the high energy exposures will be determined by fluorescamine assay and the Lowry test respectively.

3 MATERIALS AND METHODS

3.1 MATERIALS

Lysozyme, bovine serum albumin (BSA), D-glucose, fluorescamine and sodium tartrate dehydrate were all obtained from Sigma Chemicals (St-Louis, MO., USA). The remaining reagents used for the Lowry test were from Aldrich Chemical Company, Inc (New Jersey, USA), Sodium dodecyl sulphate (SDS) used for solubilizing samples that gelled upon processing was obtained from Aldrich Chemical Company, Inc. (Milkwaukee, USA) and potassium tetraborate tetrahydrate used for fluorescamine assay was obtained from Acros Organics (New Jersey, USA). Water used throughout the study was ultra-pure water obtained from the Milli-Q reagent grade water system (Millipore Corp., Bedford, MA).

3.2 EQUIPMENTS USED

Regular incubation experiments were carried out with an Isotemp® Vacuum Oven 280A (Fisher Scientific, ON, Canada). HHP experiments were carried out in an ABB Isostatic Press Model # CIP42260 (ABB Autoclave System, Autoclave Engineers, Erie, PA) with a 10 cm diameter and 55 cm height stainless steel pressure chamber. The equipment was rated for operation up to 414 MPa. Water containing a 2% water soluble oil (Autoclave Engineers, Part No. 5019, Autoclave Engineers, Erie, PA) was used as the hydrostatic fluid in which the test packages were submerged during the pressure treatment. Focused microwave irradiation experiments were carried out at atmospheric pressure with a Synthewave S402 Prolabo microwave reactor (Fontenay-Sous-Bois, France) with a mono-mode MW cavity that operated at 2450 MHz with power range of

0-300 W in a 12 cm^3 tubular quartz reactor with irradiation being monitored by PC. The temperature of reaction media was measured continuously with an IR-pyrometer, which is an integral part of the Synthewave 402. Lowry tests were carried out with a Beckman UV-Visible scanning spectrophotometer (Berkeley, CA, USA), and fluorescamine assays were carried out with a Kontron spectrofluorometer (Kontron Instruments SFM 25 spectrofluorometer, Zurich, Switzerland).

3.3 SAMPLE PREPARATION

Unless otherwise stated, the same sample preparation procedure as for the stock solution was followed for all the treatments. Two control samples were used, one did not contain any glucose and was subjected to treatments, and the other contained glucose but did not undergo any treatment.

3.3.1 LYSOZYME

Stock solution: Lysozyme (3.4303 g ± 0.0001 g) and D-glucose (0.7700 g ± 0.0001 g) were dissolved in water (5.0 mL), to give a protein to sugar molar ratio of 1:18 (approximately 1:3 ratio of lysine residues to carbonyl groups of the glucose) for the glucose-lysozyme mixture. Lysozyme (3.4015 g ± 0.0001 g) dissolved in water (5.0 mL) was used as controls that underwent treatments but did not contain any D-glucose.

Control samples of the lysozyme that did not undergo any treatment were prepared separately. Lysozyme (1.0002 g ± 0.0001 g) and D-glucose (0.2285 g ± 0.0001 g) were added to 10.0 mL of water. They were placed at -20°C in a freezer upon preparation for future analysis.

A second set of lysozyme samples with the addition of sodium azide were prepared for the incubation treatment. Lysozyme (0.9983 g ± 0.0001 g), D-glucose (0.2297 g ± 0.0001 g) and sodium azide (0.0016 g ± 0.0001 g) were dissolved in 10.0 mL of water. Controls without D-glucose that contained lysozyme (1.0228 g ± 0.0001 g) and sodium azide (0.0016 g ± 0.0001 g) were dissolved in 10.0 mL of water.

3.3.2 BSA

Stock solution: BSA (5.0430 g ± 0.0001 g) and D-glucose (2.5180 g ± 0.0001 g) were dissolved in water (19.0 mL), to give a protein to sugar molar ratio of 1:183 (approximately 1:3 ratio of lysine residues to carbonyl groups of the glucose) for the BSA-glucose mixture. BSA (5.0100 g ± 0.0001 g) dissolved in water (19.0 mL) was used as controls of the experiments that underwent treatments but without the presence of D-glucose.

Control samples of BSA that did not undergo any treatment were prepared separately. BSA (1.0004 g ± 0.0001 g) and D-glucose (0.5013 g ± 0.0001 g) was added to 10.8 mL of water. They were placed at -20°C in a freezer upon preparation for future analysis.

3.4 OVEN INCUBATION TREATMENT

Lysozyme (0.18 mL), BSA (0.575 mL) samples with D-glucose and their corresponding control samples were placed in centrifuge tubes (2.0 mL) with caps closed so as to maintain the humidity within and prevent any oxygen from entering the sample. All samples were placed in the Isotemp® Vacuum oven that had been preset to 30°C for

an hour to achieve a stable temperature. The incubation periods were 6, 8, 10, 12 days. Each of the triplicate samples was removed from the oven upon reaching the incubation time and was placed at -20°C in a freezer for later analyses.

3.5 HHP TREATMENT

Lysozyme-containing samples (0.18 mL) and BSA-containing samples (0.575 mL) were placed in plastic bags and heat sealed with oxygen removed, totaling up to 9 bags for each protein (triplicates for each of the processing time: 8 hours, 24 hours and 48 hours). The same procedure was repeated for the control samples of lysozyme and BSA.

The above samples were placed in a bigger plastic bag filled with water and was submerged into the pressure chamber. Pressure was attained at slightly above the desired processing pressure prior to submerging the samples into the chamber so as to allow for the pressure drop during processing, which can account for 1 kPsi. In our experiments, the desired processing pressure was 400 MPa (58 kPsi), and the preset pressure was 407 MPa (59 kPsi). Temperature of the chamber (30°C) was monitored by thermocouples installed within the chamber and was regulated by running hot or cold water in the jacket around the chamber.

The three processing conditions (8 hours, 24 hours, 48 hours) were performed separately, and upon reaching the processing time, the pressure of the chamber was released, with the removal of the samples from the chamber and stored at -20°C in a freezer for later analyses.

3.6 FOCUSED MICROWAVE IRRADIATION TREATMENT

3.6.1 LYSOZYME

Preliminary trials starting with the original concentration of protein and glucose were performed to determine a reasonable irradiation time range. Gels formed with dark browning were observed as the stock solutions were being microwaved for 1 minute at 50°C with varying power, therefore dilutions of the stock solutions were done. The final concentration used was diluted 1.6 times with water. The first sign of visible browning in the trial was seen at the 60-minute microwave samples. Therefore, the microwave time for the samples at 50°C with varying power started from 10 minutes to 60 minutes, with a 10-minute interval between samples, totaling up to 6 different time treatment in triplicates for both the glucose-containing samples and the controls.

Diluted samples (0.08 mL) were placed in microcentrifuge tubes, totaling up to 18 tubes for the 6 treatments in triplicates. Each sample was placed in the 12 cm^3 quartz reactor of the Prolabo unit to be microwaved for the desired period of time. Temperature was set to remain constant at 50°C with varying power, and the adapter was set to rotate clockwise during the microwave process, so as to distribute the microwave energy uniformly around the sample.

3.6.2 BSA

The above procedure was also used for BSA samples. Concentration of the BSA samples was diluted 16 times (instead of 1.6) with water, and 0.5 mL of the diluted solution placed in centrifuge tube was subjected to microwave treatment. Samples upon

microwaving for the desired processing time were stored at -20°C in a freezer for later analyses.

3.7 ANALYSES OF THE TREATED SAMPLES

Treated samples were subjected to different dilutions prior to the analyses, SDS (12%) was added to the samples that gelled upon processing to aid in solubilizing the gels for analyses. See Table 3.1 to 3.3 for the dilutions and the amount of SDS added (if necessary) to the treated glucose-containing samples and the controls of lysozyme and BSA. SDS treated samples were vortexed and centrifuged to separate the solubilized protein fraction upon the addition of SDS for analyses.

3.7.1 LOWRY TEST

The amount of soluble protein in the treated samples was detected by the Lowry method (Lowry *et al.*, 1951). Reagent A was prepared by dissolving Na_2CO_3 (100 g) in 1 L of NaOH (0.5 N); reagent B was prepared by dissolving $CuSO_4$ (10 g) in 1L of water; reagent C was prepared by dissolving sodium tartrate (20 g) in 1 L of water. Mixing of the reagents A, B and C was in the ratio of 20:1:1 in an Erlenmeyer flask (solution D). Diluted samples (30 to 80 µL, see Table 3.1 to 3.3 for amounts of individual samples used) were introduced into test tubes with water added to a total volume of 1 mL. Pure water (1 mL) was used as blank. Solution D (1 mL) was added to the samples while vortexing and let to stand at room temperature for 15 minutes. At the end of this time period, 3 mL of reagent E (10% v/v of Folin & Ciocalteu's Phenol reagent) was forcibly pipetted to the incubated solution while vortexing. The samples were then incubated for 45 minutes at room temperature and measurements were carried out on a Beckman

spectrophotometer using the wavelength at 540 nm. Three readings at different time intervals were taken and their average was used in the calculations.

3.7.2 FLUORESCAMINE ASSAY

The fluorescamine assay (Yaylayan *et al.*, 1992) was used to determine the free unreacted γ-amino groups in the samples. Between 30 to 80 μL (see Table 3.1 to 3.3 for amounts of individual samples used) samples were used for the analysis. An appropriate amount of borate buffer (pH 8.5, 0.2 M) was added to each of the samples to bring the volume to 4 mL. Fluorescamine reagent (1 mL, 15 mg in 100 mL of acetone) was rapidly added, accompanied with continuous vortexing for 5 minutes. Borate buffer (4 mL) containing no protein was used as the blank. All measurements were carried out at room temperature on a Kontron spectrofluorometer at excitation and emission wavelengths of 390 and 475 nm respectively. The instrument was calibrated using 0.01 g of quinine sulfate in 250 mL of 0.1 N of H_2SO_4. Due to the fluctuations of the readings upon placing the solutions into the spectrofluorometer, three readings were taken, at 1, 3 and 5 minutes after the 5 minutes of vortexing upon addition of fluorescamine reagent.

Curve fitting equations were generated by Microsoft® Excel (2002) software package.

Table 3.1 Dilutions of the treated samples for analysis

Lysozyme sample	Dilution factor	Volume used for test (μL)	Lysozyme control	Dilution factor	Volume used for test (μL)
CI – 0	34.3	30	CI – 6	462	50
CI – 6	441	50	CI – 8	441	50
CI – 8	441	50	CI – 10	441	50
CI – 10	462	50	CI – 12	441	50
CI – 12	427.5	50	CI[a]	34.3	30
CI[a]	34.3	30	HHP	676	80
HHP	676	80	MW	160	30
MW	160	30			
BSA sample	Dilution factor	Volume used for test (μL)	BSA control	Dilution factor	Volume used for test (μL)
CI – 0	34.3	30	CI – 6	34.3	30
CI – 6	105	30	CI – 8	105	30
CI – 8	105	30	CI – 10	105	30
CI – 12	105.	30	CI – 12	105	30

Table 3.1 shows the dilutions done and the amount of the diluted samples used for tests. 'CI' refers to samples subjected to regular incubation; 'HHP' refers to samples subjected to HHP; 'MW' refers to samples subjected to microwave irradiation. The number after the hyphen of the CI samples refers to the duration of incubation in days that the samples were subjected to. CI[a] refers to the lysozyme samples being subjected to regular incubation that contained sodium azide. Refer to Table 3.2 and 3.3 for the dilutions done on the high pressure and microwave samples of BSA for analysis.

Table 3.2 The amount of gel recovered and BSA content of HHP treated samples

Sample	Weight of gel removed from the bag (g)	Calculated amount of BSA in the gel (g)	Amount of BSA contained in 30 μL of the diluted solution (mg)
8-1	na	na	0.5860
8-2	0.6221	0.1181	0.4791
8-3	0.2596	0.0493	0.1999
24-1	0.4092	0.0777	0.3151
24-2	0.3392	0.0644	0.2612
24-3	0.1808	0.0343	0.1392
48-1	0.4135	0.0785	0.3185
48-2	0.5844	0.1110	0.4501
48-3	0.3833	0.0728	0.2952
c8-1	0.6574	0.1248	0.5063
c8-2	0.5304	0.1007	0.4085
c8-3	1.1729	0.2227	0.9033
c24-1	0.4868	0.0924	0.3749
c24-2	0.6498	0.1234	0.5004
c24-3	0.6389	0.1213	0.4921
c48-1	0.5947	0.1129	0.4580
c48-2	0.6734	0.1279	0.5186
c48-3	0.8344	0.1584	0.6426

* To all samples, 6.6 mL of water and 0.5 mL of SDS were added.

Table 3.2 shows the weight of gel recovered from the HHP treatment, and the calculated amount of BSA used for the Lowry tests and the fluorescamine assays. The sample names given designates the hours of HHP treatment that the sample had been subjected to. Sample names without "c" designate the BSA-glucose samples, and sample names with "c" refers to the control samples. The number before the hyphen refers to the hours of HHP treatment that the sample has been subjected to, and the number after the hyphen refers to the number of triplicate (e.g. 8-1 is the sample that has been subjected to 8 hours of HHP, and it is the first of the triplicate; c8-1 is the control sample is the first of the triplicate that has been subjected to 8 hours of HHP). A 30 μL of each of the diluted solutions was used for Lowry test and fluorescamine assay. See Appendix A3.1 for a sample calculation of the amount of BSA.

Table 3.3 Amount of water, SDS and the calculated BSA content in the microwave treated samples

Sample	Water added (μL)	SDS added (mL)	Amount of BSA in 30μL of the diluted sample (mg)	Sample	Water added (μL)	SDS added (mL)	Amount of BSA in 30μL of the diluted sample (mg)
10-1	9970	0.6	0.0904	c1001	700	0.6	0.7302
10-2	700	0.6	0.7350	c1002	700	0.6	0.7302
10-3	670	0.6	0.7524	c1003	700	0.6	0.7302
20-1	700	0.6	0.7350	c2001	500	0.2	1.3561
20-2	700	0.6	0.7350	c2002	700	0.6	0.7302
20-3	700	0.6	0.7350	c2003	700	0.6	0.7302
30-1	10000	-	0.0956	c3001	700	0.6	0.7302
30-2	700	0.6	0.7350	c3002	700	0.6	0.7302
30-3	700	0.6	0.7350	c3003	700	0.6	0.7302
40-1	10000	-	0.0956	c4001	700	0.6	0.7302
40-2	700	0.6	0.7350	c4002	700	0.6	0.7302
40-3	700	0.6	0.7350	c4003	700	0.6	0.7302
50-1	10000	-	0.0956	c5001	700	0.6	0.7302
50-2	700	0.6	0.7350	c5002	700	0.6	0.7302
50-3	700	0.6	0.7350	c5003	700	0.6	0.7302
60-1	10000	-	0.0956	c6001	700	0.6	0.7302
60-2	700	0.6	0.7350	c6002	1400	0.6	0.4746
60-3	700	0.6	0.7350	c6003	1400	0.6	0.4746

Table 3.3 showing the amount of water and SDS added to the gelled microwave samples, and the calculated amount of BSA in 30 μL of the diluted solution, which is the amount of the diluted solution used for Lowry tests and fluorescamine assays. The digits before the hyphen of the sample names refer to the microwave time in minutes, and the digit after the hyphen refers to the triplicate, 'c' refers to the control samples (e.g. 1001 is the sample that has been subjected to 10 minutes of microwave irradiation, and it is the first of the triplicate; while c10-1 is the control sample that has been subjected to 10 minutes of microwave and is the first of the triplicate.). See Appendix A3.1 for a sample calculation of the amount of BSA.

4 RESULTS

4.1 INTRODUCTION

All the samples of lysozyme and BSA were prepared without the addition of sodium azide except where noted (i.e. only one incubation set of lysozyme was prepared with the addition of sodium azide). Samples with a 'c' in front referred to the controls for that specific treatment. For all the sample names used, the number in front of the hyphen refers to the duration of the specific treatment that the sample has been subjected to; the number after the hyphen refers to the number of triplicate for that treatment. For incubation samples, the number before the hyphen refers to the number of **days** being incubated; for high pressure samples, it refers to the number of **hours** being pressurized; for microwave samples, it refers to the number of **minutes** being microwaved. Controls that contained D-glucose but did not undergo any treatment was day 0 sample and designated as '0' before the hyphen in the tables that contained the incubation samples. The 3 readings obtained from the Lowry test will be denoted by 1^{st}, 2^{nd} and 3^{rd} in the tables, 'of the 9X' refers to the average absorbance of the nine readings obtained from the triplicates; while the 3 readings obtained from the fluorescamine assay were denoted by '@min 1', '@min 3' and '@min 5'. Relative fluorescence determined from the fluorescamine assay was abbreviated by 'flu' in the tables. The corresponding standard deviation of the readings or calculations was designated as 'std. dev.' in the tables.

The data of the Lowry tests and the fluorescamine assays were all normalized to 0.1 mg of protein for comparison, and the standard deviations of the triplicates were calculated. All the results are tabulated in the tables below.

4.2 RESULTS OF LYSOZYME SAMPLES

4.2.1 RESULTS OF LOWRY TESTS

Table 4.1 Normalized absorbance and calculated protein content of incubated lysozyme samples that did not contain sodium azide.

	Normalized absorbance			Average normalized absorbance									
Sample	1st	2nd	3rd	1st	std.dev.	2nd	std. dev.	3rd	std. dev.	of the 9X	std. dev.	Protein content(%)	Corresponding std dev
0-1	0.820	0.830	0.831	0.886	0.06279	0.898	0.06423	0.900	0.06440	0.895	0.05568	100	6.2243
0-2	0.890	0.906	0.910										
0-3	0.946	0.958	0.959										
6-1	0.349	0.353	0.357	0.346	0.00870	0.351	0.00639	0.355	0.00845	0.350	0.00790	39.16	0.88
6-2	0.352	0.356	0.361										
6-3	0.336	0.344	0.345										
8-1	0.403	0.411	0.414	0.388	0.01335	0.397	0.01257	0.399	0.01335	0.395	0.01240	44.11	1.39
8-2	0.382	0.391	0.392										
8-3	0.379	0.388	0.390										
10-1	0.400	0.409	0.411	0.391	0.00930	0.400	0.01102	0.404	0.00966	0.398	0.01022	44.52	1.14
10-2	0.393	0.403	0.407										
10-3	0.382	0.387	0.393										
12-1	0.390	0.396	0.400	0.392	0.01203	0.399	0.01261	0.407	0.01252	0.399	0.01243	44.63	1.39
12-2	0.406	0.412	0.421										
12-3	0.382	0.387	0.399										
c6-1	0.385	0.395	0.391	0.396	0.00974	0.395	0.01288	0.392	0.01365	0.394	0.01071	44.07	1.20
c6-2	0.403	0.40	0.406										
c6-3	0.399	0.38	0.379										
c8-1	0.306	0.326	0.323	0.333	0.02611	0.341	0.01654	0.338	0.01835	0.338	0.01829	37.74	2.04
c8-2	0.337	0.338	0.333										
c8-3	0.357	0.359	0.359										
c10-1	0.314	0.314	0.308	0.313	0.00208	0.314	0.00136	0.312	0.00361	0.313	0.00227	35.01	0.25
c10-2	0.315	0.315	0.315										
c10-3	0.311	0.312	0.314										
c12-1	0.300	0.297	0.293	0.303	0.00343	0.302	0.00417	0.301	0.00630	0.302	0.00431	33.76	0.48
c12-2	0.303	0.306	0.304										
c12-3	0.307	0.303	0.304										

Table 4.2 Normalized absorbance and the calculated protein content of the lysozyme samples that contain glucose and 0.01% sodium azide being subjected to regular incubation at 30°C from day 0 to day 12

	Normalized absorbance			Average normalized absorbance								Protein content	Corresponding
Sample	1st	2nd	3rd	1st	std.dev.	2nd	std. dev.	3rd	std. dev.	of the 9X	std. dev.	(%)	std dev.
0-1	1.040	1.061	1.064	1.031	0.04530	1.053	0.04573	1.057	0.04803	1.047	0.04195	100	4.00529
0-2	1.072	1.095	1.102										
0-3	0.982	1.004	1.006										
2-1	1.035	1.055	1.058	1.026	0.01188	1.049	0.00707	1.053	0.00553	1.043	0.01497	99.58	1.42961
2-2	1.012	1.041	1.047										
2-3	1.029	1.052	1.055										
6-1	1.037	1.062	1.063	1.008	0.04306	1.031	0.04388	1.035	0.04637	1.025	0.04056	97.82	3.87239
6-2	0.958	0.981	0.981										
6-3	1.028	1.051	1.060										
8-1	1.000	1.014	1.018	0.998	0.03689	1.012	0.04287	1.015	0.04474	1.008	0.03695	96.26	3.52847
8-2	1.033	1.053	1.058										
8-3	0.959	0.968	0.969										
10-1	1.017	1.041	1.043	1.006	0.01290	1.027	0.01593	1.029	0.01607	1.021	0.01710	97.47	1.63299
10-2	0.992	1.010	1.011										
10-3	1.009	1.031	1.033										
12-1	0.916	0.926	0.926	0.993	0.07913	1.012	0.08509	1.012	0.08452	1.005	0.07246	95.99	6.91895
12-2	1.074	1.096	1.095										
12-3	0.988	1.014	1.015										

Table 4.3 Normalized absorbance and the calculated protein content of the control samples of lysozyme with 0.01% sodium azide being subjected to regular incubation from day 0 to day 12

Sample	Normalized absorbance			Average normalized absorbance								Protein content (%)	Corresponding std dev.
	1st	2nd	3rd	1st	std.dev.	2nd	std. dev.	3rd	std. dev.	of the 9X	std. dev.		
c0-1	1.075	1.082	1.087	1.094	0.05433	1.105	0.05921	1.107	0.05857	1.102	0.05008	105.23	4.78162
c0-2	1.155	1.172	1.173										
c0-3	1.052	1.060	1.061										
c2-1	1.026	1.035	1.038	0.981	0.06192	0.989	0.06674	0.990	0.06756	0.987	0.05685	94.23	5.42839
c2-2	0.910	0.913	0.913										
c2-3	1.007	1.020	1.020										
c6-1	1.007	1.021	1.026	1.016	0.07465	1.026	0.07492	1.029	0.07485	1.024	0.06505	97.78	6.21121
c6-2	0.947	0.954	0.956										
c6-3	1.095	1.104	1.106										
c8-1	1.089	1.097	1.098	1.102	0.01428	1.109	0.01303	1.110	0.01439	1.107	0.01258	105.71	1.20077
c8-2	1.118	1.122	1.126										
c8-3	1.100	1.107	1.107										
c10-1	1.062	1.075	1.074	1.043	0.02718	1.054	0.02805	1.054	0.02785	1.050	0.02465	100.29	2.35351
c10-2	1.012	1.022	1.022										
c10-3	1.054	1.065	1.066										
c12-1	1.120	1.131	1.132	1.036	0.07248	1.047	0.07248	1.048	0.07254	1.044	0.06304	99.68	6.01909
c12-2	0.998	1.002	1.002										
c12-3	0.992	1.008	1.011										

Table 4.4 Normalized absorbance and the calculated protein content of the lysozyme samples being subjected to HHP treatment from 8 hours to 48 hours at 400 MPa

	Normalized absorbance			Average normalized absorbance									
Sample	1st	2nd	3rd	1st	std.dev.	2nd	std. dev.	3rd	std. dev.	of the 9X	std. dev.	Protein content (%)	Corresponding std dev.
8-1	0.759	0.760	0.763	0.713	0.03936	0.722	0.03363	0.724	0.03409	0.720	0.03136	80.40	3.50344
8-2	0.689	0.698	0.699										
8-3	0.693	0.707	0.709										
24-1	0.700	0.715	0.716	0.696	0.00789	0.709	0.00666	0.711	0.00594	0.705	0.00900	78.82	1.00518
24-2	0.702	0.711	0.712										
24-3	0.687	0.702	0.704										
48-1	0.725	0.728	0.728	0.703	0.02027	0.708	0.01830	0.709	0.01822	0.707	0.01660	78.96	1.85507
48-2	0.700	0.706	0.707										
48-3	0.685	0.691	0.691										
c 8-1	0.774	0.787	0.788	0.763	0.01198	0.776	0.01186	0.776	0.01247	0.772	0.01230	86.25	1.37422
c 8-2	0.766	0.778	0.778										
c 8-3	0.750	0.763	0.763										
c 24-1	0.807	0.820	0.824	0.775	0.02847	0.788	0.02828	0.797	0.02372	0.786	0.02518	87.88	2.81321
c 24-2	0.766	0.780	0.787										
c 24-3	0.752	0.765	0.779										
c 48-1	0.783	0.792	0.796	0.759	0.02038	0.770	0.01934	0.773	0.02001	0.768	0.01836	85.77	2.05099
c 48-2	0.748	0.757	0.758										
c 48-3	0.748	0.761	0.766										

Table 4.5 Normalized absorbance and the calculated protein content of the lysozyme samples that contained glucose being subjected to microwave irradiation from 10 minutes to 60 minutes

	Normalized absorbance			Average normalized absorbance									
Sample	1st	2nd	3rd	1st	std.dev.	2nd	std. dev.	3rd	std. dev.	of the 9X	std. dev.	Protein content %	Corresponding std dev.
1001	0.4646	0.4799	0.4827	0.4713	0.01612	0.4904	0.01644	0.4943	0.01651	0.4853	0.01774	54.23	1.98171
1002	0.4897	0.5093	0.5132										
1003	0.4596	0.4818	0.4871										
2001	0.5122	0.5336	0.5390	0.5670	0.04926	0.5872	0.05042	0.5921	0.05027	0.5821	0.04479	65.04	5.00496
2002	0.5813	0.5944	0.5983										
2003	0.6075	0.6337	0.6389										
3001	0.5685	0.5926	0.6007	0.5919	0.03311	0.6126	0.03760	0.6179	0.03897	0.6074	0.03389	67.87	3.78699
3002	0.6298	0.6559	0.6625										
3003	0.5774	0.5892	0.5905										
4001	0.5216	0.5551	0.5645	0.5186	0.04197	0.5499	0.03693	0.5596	0.03366	0.5427	0.03752	60.64	4.19220
4002	0.4753	0.5106	0.5237										
4003	0.5591	0.5839	0.5905										
5001	0.5119	0.5368	0.5434	0.5486	0.03178	0.5739	0.03219	0.5804	0.03219	0.5676	0.03135	63.42	3.50261
5002	0.5656	0.5905	0.5970										
5003	0.5682	0.5944	0.6010										
6001	0.5805	0.6047	0.6154	0.5649	0.02439	0.5878	0.02294	0.5975	0.02162	0.5834	0.02464	65.18	2.75278
6002	0.5368	0.5617	0.5735										
6003	0.5774	0.5970	0.6036										

Table 4.6 Normalized absorbance and the calculated protein content of the control samples of lysozyme being subjected to microwave irradiation from 10 minutes to 60 minutes

	Normalized absorbance			Average normalized absorbance									
Sample	1st	2nd	3rd	1st	std.dev.	2nd	std. dev.	3rd	std. dev.	of the 9X	std. dev.	Protein content %	Corresponding std dev.
c1001	0.7082	0.7291	0.7366	0.6881	0.01753	0.7122	0.01733	0.7209	0.01726	0.7070	0.02105	79.00	2.35221
c1002	0.6760	0.6945	0.7024										
c1003	0.6800	0.7130	0.7236										
c2001	0.6044	0.6166	0.6206	0.6869	0.07215	0.7007	0.07319	0.7055	0.07389	0.6977	0.06384	77.96	7.13305
c2002	0.7381	0.7500	0.7552										
c2003	0.7183	0.7354	0.7407										
c3001	0.5868	0.6206	0.6341	0.6010	0.01496	0.6232	0.00824	0.6321	0.00811	0.6188	0.01682	69.14	1.87881
c3002	0.5994	0.6166	0.6232										
c3003	0.6166	0.6325	0.6391										
c4001	0.6152	0.6422	0.6517	0.5519	0.15278	0.5723	0.15979	0.5799	0.16653	0.5680	0.13896	63.47	15.52617
c4002	0.3776	0.3895	0.3895										
c4003	0.6628	0.6853	0.6985										
c5001	0.6153	0.6377	0.6523	0.6413	0.05438	0.6619	0.06114	0.6707	0.05772	0.6580	0.05176	73.52	5.78276
c5002	0.7038	0.7315	0.7354										
c5003	0.6047	0.6166	0.6245										
c6001	0.8126	0.8491	0.8640	0.7106	0.10263	0.7359	0.11497	0.7475	0.11915	0.7313	0.09877	81.71	11.03548
c6002	0.7117	0.7394	0.7526										
c6003	0.6074	0.6192	0.6258										

4.2.2 RESULTS OF FLUORESCAMINE ASSAYS

Table 4.7 The calculated extent of glycation or denaturation of the lysozyme incubated samples without sodium azide from day 0 to day 12

Sample	Normalized relative fluorescance (Flu.) @min 1	@min 3	@min 5	Average flu of triplicate	std. dev.	Flu content %	% drop in flu	Amt protein from Lowry test (mg)	Expected fluorescence from Lowry	Difference in fluorescence	# lysyl groups got Glycated or denatured	Average #of reacted lysyl groups	Extent of glycation or denaturation (%)	Corresponding std dev.
0-1	112.4	120.8	122.7	117.5	6.34991	100.00	-	0.1000	117.500	0	0	0	0	6.34991
0-2	106.3	113.5	114.5											
0-3	117.3	125.5	124.2											
6-1	20.3	21.2	21.2	21.8	1.28196	18.55	81.45	0.0392	46.000	24.214	8.65E-09	1.24	20.61	1.21261
6-2	22.6	23.4	24.1											
6-3	20.7	21.2	21.2											
8-1	25.3	26.9	26.8	26.6	0.85255	22.67	77.33	0.0441	51.815	25.187	8.99E-09	1.29	21.44	0.68633
8-2	27.1	27.9	27.6											
8-3	25.7	26.1	26.3											
10-1	22.5	23.4	23.1	23.0	2.98966	19.55	80.45	0.0445	52.297	29.331	1.05E-08	1.50	24.96	3.24960
10-2	19.3	19.8	19.4											
10-3	25.8	26.8	26.5											
12-1	24.5	24.4	24.5	23.5	1.10622	20.00	80.00	0.0446	52.426	28.923	1.03E-08	1.48	24.62	1.15918
12-2	23.4	24.1	24.1											
12-3	21.6	22.4	22.3											
c6-1	43.2	45.0	44.3	42.2	2.20501	35.89	64.11	0.0441	51.768	9.607	3.43E-09	0.49	8.18	0.42763
c6-2	41.5	43.7	42.9											
c6-3	38.7	40.6	39.5											
c8-1	37.0	38.2	37.9	36.9	1.14588	31.45	68.55	0.0377	44.332	7.394	2.64E-09	0.38	6.29	0.19520
c8-2	36.7	38.3	37.1											
c8-3	34.8	36.3	36.0											
c10-1	34.3	35.1	34.8	33.8	1.29109	28.81	71.19	0.0350	41.125	7.280	2.60E-09	0.37	6.20	0.23636
c10-2	34.0	35.1	34.8											
c10-3	32.3	32.5	31.8											
c12-1	27.4	29.1	28.0	27.7	0.60436	23.57	76.43	0.0338	39.657	11.968	4.27E-09	0.61	10.19	0.22233
c12-2	27.2	27.7	27.3											
c12-3	27.4	28.0	27.2											

Table 4.8 The calculated extent of glycation of lysozyme samples that contained glucose and 0.01% sodium azide being subjected to regular incubation from day 0 to day 12

Sample	Normalized relative fluorescence (Flu) @min 1	@min 3	@min 5	Average flu of triplicate	std. dev.	Flu Content %	% drop in flu	Amt protein from Lowry test (mg)	Expected fluorescence from Lowry	Difference in fluorescence	# lysyl groups that got glycated	Average # of reacted lysyl groups	Extent of glycation (%)	Corresponding std dev.
0-1	67.0	70.8	70.6	73.6	5.44594	100.00	-	0.1000	73.600	-	-	-	-	5.44594
0-2	76.0	82.1	82.1											
0-3	68.7	73.2	71.8											
2-1	74.5	78.4	78.3	69.1	10.45026	93.88	6.12	0.0996	73.289	4.211	2.40E-09	0.34	5.72	0.86550
2-2	71.8	76.1	76.3											
2-3	53.9	56.7	55.6											
6-1	67.1	70.8	70.1	69.2	1.63144	94.06	5.94	0.0978	71.965	2.752	1.57E-09	0.22	3.74	0.08814
6-2	66.9	70.5	69.5											
6-3	67.3	70.4	70.4											
8-1	71.0	76.1	74.9	68.1	5.32357	92.53	7.47	0.0963	70.861	2.775	1.58E-09	0.23	3.77	0.29477
8-2	66.5	68.7	68.5											
8-3	60.6	63.4	63.1											
10-1	69.4	72.6	71.9	68.8	2.68465	93.44	6.56	0.0975	71.744	2.987	1.70E-09	0.24	4.06	0.15848
10-2	66.2	70.7	68.4											
10-3	64.4	67.8	67.2											
12-1	53.2	56.4	56.0	66.9	9.59757	90.87	9.13	0.0960	70.640	3.774	2.15E-09	0.31	5.13	0.73602
12-2	73.5	78.2	78.3											
12-3	66.7	70.7	68.8											

Table 4.9 The calculated extent of denaturation of control samples of lysozyme that contained 0.01% sodium azide when being subjected to regular incubation from day 0 to day 12

Sample	Normalized relative fluorescence (Flu) @min 1	@min 3	@min 5	Average flu. of triplicate	std. dev.	Flu content %	% drop in flu	Amt protein from Lowry test (mg)	Expected fluorescence from Lowry	Difference in fluorescence	# lysyl groups that got denatured	Average # of reacted lysyl groups	Extent of denaturation (%)	Corresponding std dev.
c0-1	70.8	73.6	73.3	73.9	3.96447	100.40	-0.40	0.1052	77.410	3.534	2.01E-09	0.29	4.80	0.25770
c0-2	74.9	79.0	80.4											
c0-3	67.4	72.8	72.7											
c2-1	68.9	72.8	72.6	69.4	5.49535	94.30	5.70	0.0942	69.316	-0.071	-4.05E-11	-0.01	-0.10	-0.00765
c2-2	61.6	63.5	62.5											
c2-3	72.7	76.4	73.5											
c6-1	69.7	73.3	69.8	71.2	3.27787	96.79	3.21	0.0978	71.965	0.746	4.25E-10	0.06	1.01	0.04664
c6-2	66.2	68.8	69.4											
c6-3	72.8	76.9	74.1											
c8-1	72.9	76.9	76.1	68.6	5.62787	93.18	6.82	0.1057	77.778	9.216	5.25E-09	0.75	12.52	1.02778
c8-2	61.0	63.0	65.5											
c8-3	65.4	67.7	68.5											
c10-1	70.0	74.0	74.1	70.1	2.72877	95.29	4.71	0.1003	73.804	3.685	2.10E-09	0.30	5.01	0.19483
c10-2	66.0	69.8	68.3											
c10-3	69.0	71.8	68.1											
c12-1	72.4	75.9	76.7	69.2	4.90972	93.98	6.02	0.0997	73.363	4.212	2.40E-09	0.34	5.72	0.40628
c12-2	62.8	65.5	65.1											
c12-3	66.0	68.8	69.3											

Table 4.10 The calculated extent of glycation or denaturation of lysozyme samples being subjected to HHP from 8 hour to 48 hours at 400 MPa

Sample	Normalized relative fluorescence (Flu) @min1	@min3	@min5	Average flu. of the triplicate	std. dev.	Flu %	% drop in flu %	Amt protein from Lowry test (mg)	Expected fluorescence from Lowry	Difference in fluorescence	# lysyl groups got glycated or denatured	Average # of reacted lysyl groups	Extent of Glycation or denaturation (%)	Corresponding std dev
8-1	46.7	48.0	48.7	47.3	1.18315	40.213	59.79	0.0804	113.819	66.569	2.38E-08	3.40	56.65	1.41862
8-2	46.3	49.0	47.2											
8-3	45.5	47.6	46.3											
24-1	45.0	47.5	46.2	46.1	1.33484	39.208	60.79	0.0788	92.614	46.544	1.66E-08	2.38	39.61	1.14774
24-2	45.1	47.6	47.3											
24-3	43.6	46.0	46.4											
48-1	50.8	52.6	50.8	49.6	2.14915	42.212	57.79	0.0790	92.778	43.179	1.54E-08	2.20	36.75	1.59233
48-2	48.6	51.3	50.3											
48-3	45.6	48.2	48.0											
c 8-1	54.9	58.3	57.4	56.4	1.81247	47.986	52.01	0.0863	101.344	44.960	1.61E-08	2.30	38.26	1.23002
c 8-2	54.0	57.5	57.8											
c 8-3	53.3	57.0	57.3											
c 24-1	59.1	62.2	61.6	57.0	3.34513	48.493	51.51	0.0879	103.259	46.280	1.65E-08	2.36	39.39	2.31236
c 24-2	54.5	57.5	56.1											
c 24-3	53.1	54.9	53.9											
c 48-1	50.6	52.9	51.6	49.1	3.92102	41.793	58.21	0.0858	100.780	51.673	1.85E-08	2.64	43.98	3.51143
c 48-2	49.8	53.5	51.0											
c 48-3	42.6	45.4	44.6											

Table 4.11 The calculated extent of glycation of lysozyme samples that contained glucose being subjected to microwave irradiation from 10 minutes to 60 minutes

Sample	Normalized Relative fluorescence (Flu) @min1	@min3	@min5	Average flu. of the triplicate	std. dev.	Flu content %	% drop in flu	Amt protein from Lowry test (mg)	Expected fluorescence from Lowry	Difference in fluorescence	# lysyl groups got glycated	Average # of reacted lysyl groups	Extent of glycation (%)	Corresponding std dev
1001	25.9	27.1	26.7	26.0	1.17234	22.15	77.85	0.0542	63.720	37.697	1.35E-08	1.92	32.08	1.44535
1002	26.3	27.4	27.0											
1003	24.1	25.1	24.6											
2001	28.6	29.5	29.6	29.6	1.32124	25.18	74.82	0.0650	76.422	46.836	1.67E-08	2.39	39.86	1.78010
2002	27.6	29.2	28.5											
2003	31.9	30.8	30.5											
3001	19.8	20.2	20.0	29.0	7.07686	24.67	75.33	0.0679	79.747	50.757	1.81E-08	2.59	43.20	10.54522
3002	35.0	36.4	36.3											
3003	29.7	31.9	31.6											
4001	30.2	32.4	31.2	30.4	1.05325	25.91	74.09	0.0606	71.252	40.807	1.46E-08	2.08	34.73	1.20144
4002	29.6	31.2	30.1											
4003	28.8	30.1	30.4											
5001	27.8	28.0	28.4	28.5	0.55462	24.27	75.73	0.0634	74.519	46.005	1.64E-08	2.35	39.15	0.76159
5002	28.0	28.5	28.7											
5003	28.5	29.5	29.2											
6001	34.5	35.3	35.4	30.8	3.59080	26.18	73.82	0.0652	76.587	45.821	1.64E-08	2.34	39.00	4.55142
6002	29.5	31.0	30.6											
6003	26.2	27.0	27.5											

Table 4.12 The calculated extent of denaturation of lysozyme controls being subjected to microwave irradiation from 10 minutes to 60 minutes.

Sample	Normalized relative fluorescence (Flu) @min 1	@min3	@min5	Average flu of the triplicate	std. dev.	Flu content %	% drop in flu	Amt protein from Lowry test (mg)	Expected fluorescence from Lowry	Difference in fluorescence	# lysyl groups got denatured	Average # reacted lysyl groups	Extent of denaturation (%)	Corresponding std dev
c1001	37.0	39.2	38.0	38.5	0.80743	32.76	67.24	0.0790	92.825	54.326	1.94E-08	2.77	46.24	0.96969
c1002	39.0	39.5	39.0											
c1003	37.9	38.0	39.0											
c2001	33.3	33.7	33.9	40.3	5.25772	34.31	65.69	0.0780	91.603	51.289	1.83E-08	2.62	43.65	5.69283
c2002	41.3	43.2	41.5											
c2003	44.8	45.6	45.7											
c3001	30.2	31.4	31.5	30.6	1.59942	26.08	73.92	0.0691	81.240	50.599	1.81E-08	2.58	43.06	2.24789
c3002	27.2	29.8	29.8											
c3003	31.3	32.3	32.2											
c4001	29.3	29.6	28.7	30.2	11.18030	25.70	74.30	0.0635	74.577	44.377	1.58E-08	2.27	37.77	13.98146
c4002	17.4	18.1	18.0											
c4003	43.0	43.7	44.0											
c5001	35.8	37.5	36.8	37.5	3.99692	31.93	68.07	0.0735	86.386	48.873	1.75E-08	2.50	41.59	4.43177
c5002	42.0	42.6	42.6											
c5003	32.6	33.9	33.7											
c6001	48.8	52.5	50.7	41.5	8.29433	35.30	64.70	0.0817	96.009	54.535	1.95E-08	2.78	46.41	9.28198
c6002	41.1	42.8	42.4											
c6003	31.3	32.3	31.4											

4.3 RESULTS OF BSA SAMPLES

4.3.1 RESULTS OF LOWRY TESTS

Table 4.13 Normalized absorbance and calculated protein content of BSA samples subjected to regular incubation

	Normalized absorbance			Average normalized absorbance									
Sample	1st	2nd	3rd	1st	std. dev.	2nd	std. dev.	3rd	std. dev.	of the 9X	std. dev.	Protein content %	Corresponding std dev
0-1	0.578	0.593	0.595	0.568	0.00904	0.586	0.00681	0.585	0.00857	0.580	0.01105	100.00	1.90687
0-2	0.560	0.580	0.583										
0-3	0.568	0.583	0.578										
6-1	0.545	0.559	0.563	0.575	0.02830	0.583	0.02191	0.591	0.02621	0.583	0.02318	100.57	3.99854
6-2	0.602	0.602	0.615										
6-3	0.578	0.589	0.593										
8-1	0.580	0.591	0.591	0.575	0.02714	0.585	0.02788	0.590	0.02611	0.583	0.02437	100.60	4.20388
8-2	0.545	0.555	0.563										
8-3	0.599	0.610	0.615										
10-1	0.519	0.534	0.534	0.562	0.03693	0.573	0.03331	0.573	0.03334	0.569	0.03044	98.17	5.25036
10-2	0.581	0.592	0.593										
10-3	0.585	0.592	0.591										
12-1	0.584	0.589	0.588	0.538	0.05741	0.545	0.05510	0.549	0.05088	0.544	0.04747	93.91	8.18834
12-2	0.558	0.563	0.569										
12-3	0.474	0.484	0.492										
c6-1	0.506	0.524	0.517	0.425	0.07108	0.432	0.08076	0.430	0.07617	0.429	0.06599	73.96	11.38224
c6-2	0.376	0.376	0.376										
c6-3	0.391	0.394	0.397										
c8-1	0.531	0.538	0.541	0.487	0.09016	0.494	0.08891	0.494	0.09041	0.492	0.07787	84.80	13.43300
c8-2	0.546	0.552	0.552										
c8-3	0.383	0.391	0.390										
c10-1	0.613	0.610	0.610	0.509	0.11741	0.513	0.11415	0.514	0.11433	0.512	0.09988	88.33	17.22842
c10-2	0.534	0.542	0.544										
c10-3	0.382	0.387	0.387										
c12-1	0.582	0.588	0.586	0.498	0.10598	0.503	0.10675	0.499	0.11268	0.500	0.09400	86.26	16.21499
c12-2	0.533	0.538	0.539										
c12-3	0.379	0.383	0.372										

Table 4.14 Normalized absorbance and the calculated protein content of BSA samples that were being subjected to HHP from 8 hours to 48 hours at 400 MPa

	Normalized absorbance			Average normalized absorbance									
Sample	1st	2nd	3rd	1st	std.dev.	2nd	std.dev	3rd	std.dev.	of the 9X	std.dev.	Protein content %	Corresponding std.dev
8-1	0.179	0.190	0.191	0.172	0.11478	0.185	0.124505	0.189	0.12899	0.182	0.10674	31.3852	18.4027
8-2	0.053	0.058	0.060										
8-3	0.283	0.307	0.318										
24-1	0.177	0.190	0.196	0.200	0.02024	0.216	0.022941	0.222	0.02197	0.213	0.02112	36.6672	3.6410
24-2	0.210	0.225	0.231										
24-3	0.214	0.233	0.237										
48-1	0.122	0.132	0.136	0.122	0.00059	0.132	0.000020	0.135	0.00102	0.129	0.00615	22.3089	1.0609
48-2	0.121	0.132	0.134										
48-3	0.163	0.173	0.179										
c8-1	0.050	0.052	0.053	0.060	0.02682	0.063	0.028234	0.065	0.02904	0.063	0.02437	10.8469	4.2022
c8-2	0.091	0.096	0.098										
c8-3	0.041	0.043	0.044										
c24-1	0.063	0.066	0.068	0.071	0.01304	0.075	0.013739	0.077	0.01416	0.074	0.01211	12.8172	2.0877
c24-2	0.064	0.068	0.070										
c24-3	0.086	0.091	0.093										
c48-1	0.028	0.029	0.029	0.036	0.00708	0.037	0.007357	0.038	0.00835	0.037	0.00666	6.4300	1.1475
c48-2	0.040	0.041	0.043										
c48-3	0.040	0.042	0.043										

Table 4.15 Normalized absorbance and the calculated protein content of BSA samples containing glucose that were being subjected to microwave irradiation from 10 minutes to 60 minutes

	Normalized absorbance			Average normalized absorbance.									
Sample	1st	2nd	3rd	1st	std.dev.	2nd	std.dev	3rd	std.dev.	of the 9X	std.dev.	Protein content %	Corresponding std dev
1001	0.216	0.228	0.233	0.176	0.04073	0.185	0.04326	0.188	0.04467	0.183	0.03759	31.52	6.48064
1002	0.134	0.141	0.144										
1003	0.177	0.185	0.187										
2001	0.135	0.141	0.144	0.148	0.03214	0.155	0.03245	0.158	0.03224	0.154	0.02826	26.48	4.87263
2002	0.125	0.132	0.134										
2003	0.185	0.192	0.194										
3001	0.031	0.041	0.039	0.091	0.05797	0.097	0.05560	0.097	0.05716	0.095	0.04939	16.38	8.51634
3002	0.147	0.152	0.153										
3003	0.094	0.098	0.100										
4001	0.016	0.013	0.012	0.082	0.05784	0.084	0.06186	0.084	0.06317	0.083	0.05283	14.39	9.10912
4002	0.112	0.115	0.116										
4003	0.119	0.124	0.125										
5001	0.019	0.018	0.018	0.057	0.05074	0.058	0.05216	0.058	0.05309	0.057	0.04504	9.90	7.76573
5002	0.114	0.117	0.118										
5003	0.037	0.038	0.038										
6001	0.104	0.111	0.116	0.082	0.04625	0.086	0.04783	0.089	0.05028	0.085	0.04181	14.74	7.20785
6002	0.029	0.031	0.031										
6003	0.113	0.116	0.119										

Table 4.16 Normalized absorbance and the calculated protein content of BSA control samples that were being subjected to microwave irradiation from 10 minutes to 60 minutes

	Normalized absorbance			Average normalized absorbance									
Sample	1st	2nd	3rd	1st	std.dev.	2nd	std.dev	3rd	std.dev.	of the 9X	std.dev.	Protein content %	Corresponding std dev
c1001	0.012	0.012	0.012	0.028	0.02530	0.030	0.02630	0.030	0.02688	0.029	0.02267	5.05	3.90874
c1002	0.016	0.017	0.016										
c1003	0.058	0.060	0.061										
c2001	0.027	0.028	0.028	0.035	0.01164	0.036	0.01217	0.036	0.01232	0.036	0.01046	6.17	1.80326
c2002	0.029	0.030	0.030										
c2003	0.048	0.050	0.051										
c3001	0.041	0.042	0.043	0.036	0.01751	0.038	0.01814	0.038	0.01857	0.037	0.01568	6.45	2.70273
c3002	0.017	0.018	0.018										
c3003	0.051	0.053	0.054										
c4001	0.015	0.016	0.016	0.017	0.00283	0.017	0.00294	0.017	0.00288	0.017	0.00251	2.93	0.43256
c4002	0.015	0.015	0.015										
c4003	0.020	0.021	0.020										
c5001	0.011	0.012	0.011	0.013	0.00445	0.013	0.00484	0.013	0.00531	0.013	0.00425	2.26	0.73282
c5002	0.009	0.010	0.01										
c5003	0.018	0.019	0.019										
c6001	0.018	0.018	0.018	0.019	0.00097	0.020	0.00146	0.020	0.00165	0.020	0.00127	3.38	0.21969
c6002	0.020	0.021	0.021										
c6003	0.019	0.020	0.020										

4.3.2 RESULTS OF FLUORESCAMINE ASSAYS

Table 4.17 The calculated extent of glycation or denaturation of BSA samples being subjected to incubation from 0 to 12 days

Sample	Normalized relative absorbance (Flu) @min1	@min3	@min5	Average flu of the triplicate	std. dev.	Flu content %	% drop in flu	Amt protein from Lowry test (mg)	Expected fluorescence from Lowry	Difference in fluorescence	# lysyl groups got glycated or denatured	Avg. # of reacted lysyl groups	extent of glycation or denaturation (%)	Corresponding std dev.
0-1	109.5	113.3	113.3	109.2	3.53051	100.00	-	0.1000	109.233	-	-	-	-	3.53051
0-2	104.8	108.4	113.3											
0-3	103.9	108.1	108.8											
6-1	75.0	77.6	78.4	73.8	7.52474	67.54	32.46	0.1006	109.888	36.109	3.05529E-08	20.16	33.06	3.37152
6-2	62.8	64.7	64.8											
6-3	78.2	80.6	81.9											
8-1	74.3	76.9	77.1	73.0	2.76076	66.87	33.13	0.1006	109.888	36.842	3.11728E-08	20.57	33.73	1.27474
8-2	68.7	71.2	71.7											
8-3	71.2	72.9	73.5											
10-1	60.4	62.0	62.0	68.2	5.21802	62.44	37.56	0.0982	107.267	39.059	3.30483E-08	21.81	35.76	2.73547
10-2	69.4	72.1	71.8											
10-3	70.5	72.3	73.5											
12-1	72.3	73.5	74.9	66.8	6.01257	61.14	38.86	0.0939	102.570	35.781	3.0275E-08	19.98	32.76	2.94887
12-2	58.4	60.2	61.3											
12-3	65.2	67.9	67.6											
c6-1	83.2	86.1	86.1	71.6	10.89651	65.53	34.47	0.0740	80.832	9.252	7.82865E-09	5.17	8.47	1.28943
c6-2	60.8	60.4	60.1											
c6-3	68.4	69.4	69.4											
c8-1	86.3	87.1	87.4	80.5	6.25278	73.69	26.31	0.0848	92.630	12.139	1.02707E-08	6.78	11.11	0.86326
c8-2	81.0	82.3	82.1											
c8-3	71.9	73.1	73.1											
c10-1	99.3	99.7	100.4	86.8	13.65795	79.48	20.52	0.0883	96.453	9.632	8.14968E-09	5.38	8.82	1.38712
c10-2	91.1	91.5	91.5											
c10-3	68.6	69.6	69.7											
c12-1	100.8	100.7	101.9	81.4	14.79963	74.55	25.45	0.0863	94.268	12.840	1.08641E-08	7.17	11.75	2.13640
c12-2	70.7	71.6	73.0											
c12-3	70.1	72.2	71.9											

Table 4.18 The calculated extent of glycation or denaturation of BSA samples that were being subjected to HHP from 8 hours to 48 hours at 400 MPa

Sample	Normalized relative fluorescence (Flu) @min1	@min3	@min5	Average flu. of the triplicate	std.dev.	Flu content %	% drop in flu	Amt protein from Lowry test (mg)	Expected fluorescence from Lowry	Difference in fluorescence	# lysyl groups that got glycated or denatured	Average # of reacted lysyl groups	Extent of glycation or denaturation (%)	Corresponding std dev.
8-1	13.6	13.8	13.8	17.84	3.41274	16.33	83.67	0.0314	34.299	16.458	1.39251E-08	9.19	15.07	2.88193
8-2	17.5	18.4	19.2											
8-3	21.3	21.3	21.8											
24-1	16.5	17.4	16.2	14.25	2.80367	13.04	86.96	0.0367	40.089	25.841	2.18649E-08	14.43	23.66	4.65545
24-2	14.8	15.5	15.9											
24-3	10.3	10.6	10.9											
48-1	7.9	8.2	8.3	7.69	0.52737	7.04	92.96	0.0223	24.359	16.670	1.41049E-08	9.31	15.26	1.04673
48-2	7.0	7.3	7.4											
48-3	11.5	13.9	12.1											
c8-1	2.4	2.5	2.5	3.74	1.59454	3.43	96.57	0.0108	11.797	8.056	6.81623E-09	4.50	7.37	3.14319
c8-2	5.5	6.4	5.5											
c8-3	2.8	3.0	3.1											
c24-1	3.5	3.6	3.5	4.55	0.96848	4.17	95.83	0.0128	13.982	9.431	7.97963E-09	5.27	8.63	1.83731
c24-2	5.7	5.8	5.7											
c24-3	4.4	4.4	4.3											
c48-1	2.8	3.0	3.0	2.40	0.53353	2.19	97.81	0.0064	6.991	4.593	3.88663E-09	2.57	4.21	0.93583
c48-2	2.5	2.6	2.5											
c48-3	1.7	1.7	1.7											

Table 4.19 The calculated extent of glycation of BSA samples which contained glucose when being subjected to microwave irradiation from 10 minutes to 60 minutes

Sample	Normalized relative fluorescence (Flu) @min1	@min3	@min5	Average flu. of the triplicate	std.dev.	Flu content (%)	% drop in flu	Amt protein from Lowry test (mg)	Expected fluorescence from Lowry	Difference in fluorescence	# lysyl groups that got glycated	Average # of reacted lysyl groups	Extent of glycation (%)	Corresponding std dev.
1001	14.5	13.5	10.2	16.24	3.12483	14.87	85.13	0.0316	34.518	18.277	1.55E-08	10.21	16.733	3.21957
1002	16.2	16.9	16.8											
1003	18.8	19.6	19.8											
2001	15.3	12.9	12.7	13.22	1.02775	12.10	87.90	0.0265	28.947	15.731	1.33E-08	8.78	14.402	1.12000
2002	14.1	13.6	13.4											
2003	12.0	12.4	12.5											
3001	2.8	3.7	3.7	8.35	5.35067	7.64	92.36	0.0164	17.914	9.565	8.09E-09	5.34	8.757	5.61215
3002	14.8	15.4	15.5											
3003	6.9	6.2	6.1											
4001	2.0	2.2	2.2	7.03	3.71051	6.44	93.56	0.0144	15.730	8.699	7.36E-09	4.86	7.963	4.20255
4002	8.7	9.1	9.1											
4003	9.9	10.1	10.1											
5001	2.1	2.4	2.3	5.63	4.52365	5.16	94.84	0.0099	10.814	5.180	4.38E-09	2.89	4.742	3.80795
5002	11.3	11.8	11.9											
5003	2.9	3.0	3.0											
6001	2.8	3.2	2.8	4.12	2.94121	3.78	96.22	0.0147	16.057	11.933	1.01E-08	6.66	10.924	7.78950
6002	1.4	1.5	1.5											
6003	7.9	7.9	8.1											

Table 4.20 The calculated extent of denaturation of BSA controls when being subjected to microwave irradiation from 10 minutes to 60 minutes

Sample	Normalized relative fluorescence (Flu) @min1	@min3	@min5	Average flu. of the triplicate	std.dev.	Flu content (%)	% drop in flu	Amt protein from Lowry test (mg)	Expected fluorescence from Lowry	Difference in fluorescence	# lysyl groups that got denatured	Average # reacted lysyl groups	Extent of denaturation (%)	Corresponding std dev.
c1001	1.2	1.2	1.3	2.29	1.15831	2.10	97.90	0.0050	5.462	3.172	2.68E-09	1.77	2.904	1.46940
c1002	2.8	2.7	2.7											
c1003	3.3	3.4	3.4											
c2001	1.5	1.6	1.6	1.98	0.61346	1.81	98.19	0.0062	6.772	4.791	4.05E-09	2.68	4.386	1.35773
c2002	1.5	1.6	1.6											
c2003	2.8	2.8	2.8											
c3001	2.9	3.0	3.0	2.82	0.64830	2.58	97.42	0.0064	6.991	4.170	3.53E-09	2.33	3.817	0.87723
c3002	3.4	3.5	3.6											
c3003	2.0	2.0	2.1											
c4001	1.5	1.5	1.6	1.53	0.05260	1.40	98.60	0.0029	3.168	1.638	1.39E-09	0.92	1.500	0.05159
c4002	1.5	1.6	1.6											
c4003	1.4	1.5	1.5											
c5001	1.1	1.2	1.2	1.16	0.38125	1.07	98.93	0.0023	2.512	1.348	1.14E-09	0.75	1.234	0.40426
c5002	0.7	0.7	0.7											
c5003	1.6	1.6	1.6											
c6001	5.8	6.2	6.4	3.46	2.01354	3.17	96.83	0.0034	3.714	0.255	2.16E-10	0.14	0.234	0.13616
c6002	2.1	1.9	1.9											
c6003	2.1	2.3	2.4											

5 DISCUSSION

5.1 EFFECT OF HHP AND MICROWAVE IRRADIATION ON LYSOZYME

Samples subjected to HHP and microwave irradiation did not contain sodium azide, all discussion in this section is referred to systems without sodium azide, unless otherwise specified.

5.1.1 LOWRY TEST

Figure 5.1 shows the percentage of water soluble lysozyme remaining after incubation for a period of 12 days. About 40% of the protein content was retained when the lysozyme-glucose mixture was incubated for 6 days, then it increased to about 44% and remained relatively constant as the incubation period increased. Lysozyme is well known as an effective antimicrobial agent in food (Ibrahim *et al.*, 1997; Bower *et al.*, 1998). Its activity involves cleavage of glycosidic bonds in the bacterial peptidoglycan, thereby puncturing the cell walls (Sofos *et al.*, 1998). The big drop in the lysozyme concentration upon incubation at 30°C suggests that the lysozyme could be acting upon micro-organisms that present in the solution, therefore limiting the tryptophan and tyrosine residues of the protein. Other possible explanation for the dramatic drop in protein content is that the protein was undergoing internal crosslinking that involved the tryptophan and tyrosine residues. However, this could be reversible and as the incubation time increased, the lysozyme-glucose complex released the tryptophan and/or tyrosine residues and could be detected by the Lowry reagent. This agrees with the reported amino acid composition of egg albumin with glucose. Tanaka *et al.* (1977) reported that the percentage of tyrosine of the egg albumin-glucose mixture was comparatively lower

when incubated for 6 days compared with 3 and 10 day incubations. This accounts for the lower absorbance detected by the Lowry reagent. Protein loss of the lysozyme-glucose mixture was stabilized at around 55% from day 8 onwards. On the other hand, the protein content of the controls continuously decreased as the duration of incubation increased. This suggests that lysozyme by itself may have undergone irreversible structural changes upon incubation, with the amino acids crosslinking with each other irreversibly. In other words, this suggests that glucose acts as a protectant or a shield to the protein in protecting the amino acids from being modified or further crosslink, which agrees with a previous study (Prabhakaram and Ortwerth, 1994). In addition, the protein loss for incubated samples was high overall. This could also be due to the oxygen catalyzed free radical damage or degradation of the protein.

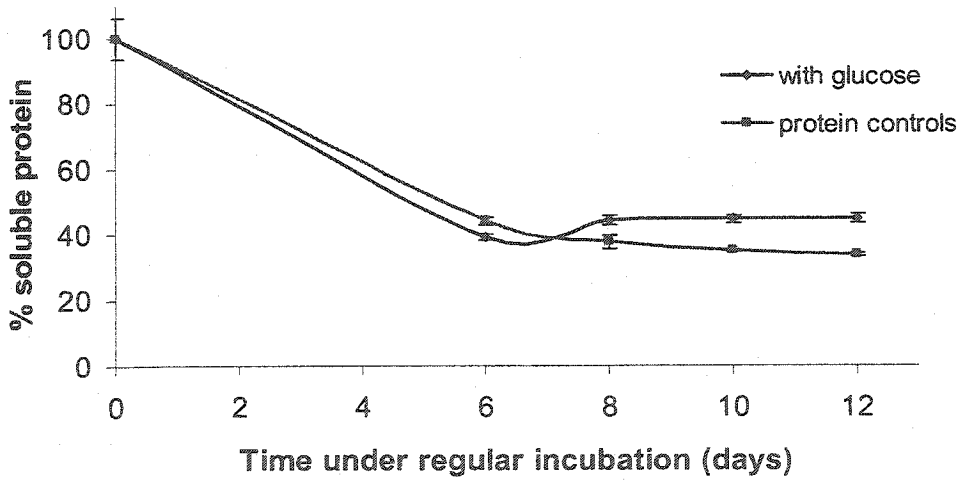


Figure 5.1 Percentage of water soluble lysozyme remained in the samples upon being subjected to regular incubation at 30°C over a period of 12 days, as compared to the initial amount of protein at day 0.

Comparing the above result with lysozyme samples that have been incubated in the presence of sodium azide (Figure 5.2), it becomes clear that micro-organisms did play

a role in degrading the lysozyme upon prolonged exposures and limiting its availability with negligible decrease in the protein content for both the glucose-containing samples and the protein controls that contained sodium azide, in which, sodium azide is commonly used as an effective antimicrobial agent (Bower *et al.*, 1998).

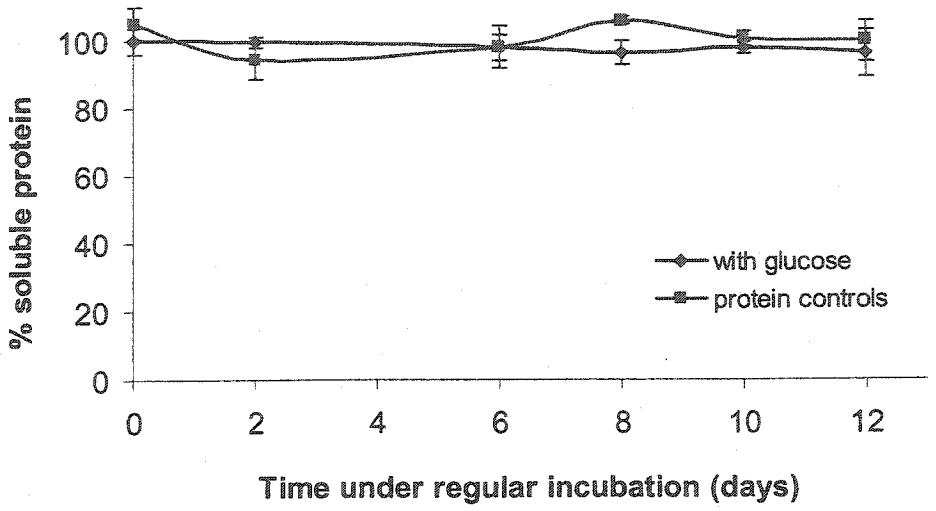


Figure 5.2 Percentage of water soluble lysozyme remaining in the samples (that contained 0.01% sodium azide) upon being subjected to regular incubation at 30°C over a period of 12 days, as compared to the initial amount of protein at day 0.

Lysozyme samples, when subjected to 400 MPa of HHP at 30°C, encounter less damage when compared to the samples subjected to regular incubation treatment (Figure 5.3). Pressure treated glucose-containing samples exhibited a 20% drop in protein content; while for the protein controls, there was only around a 15% drop. Lysozyme has been reported to be pressure resistant and only shows slight changes in the structure when the protein is subjected to 300 MPa for 30 minutes (Tedford *et al.*, 1999); as well as when lysozyme is subjected to 200 MPa for 7 days (Webb *et al.*, 2000). The higher pressure and the longer duration of pressurization applied in our experiment could explain for the

15 to 20% loss in protein content, in which some of the secondary and the tertiary structures of the protein got disrupted.

Microwave-treated samples also encountered less damage to the protein structure as compared to those oven-incubated without sodium azide (Figure 5.4). Protein content of the microwave-treated glucose containing samples had a higher loss when compared with that of the controls. Around 20 to 30% of protein was lost for the lysozyme controls; while the lysozyme-glucose mixture encountered a loss of up to 40 to 55%. The highest drop in protein content occurred for lysozyme-glucose samples subjected to 10 minutes of microwaving. However, the protein content increased slightly as the microwave time increased thereafter. This could be due to the reversibility of ARP as the protein underwent longer exposures to microwave irradiation. In the absence of glucose, the control lysozyme samples also showed fluctuations in the amount of soluble protein as the microwave time increased. In any of the two cases for the lysozyme samples that contained glucose and the lysozyme controls, hydrogen bonds could form between N-H and C=O groups of adjacent turns of the peptide spirals, and also between phenolic OH groups and the carbonyl groups (Haurowitz, 1963). The formation of these crosslinks could be reversible over time, and tryptophan and tyrosine residues could involve in the formation of these crosslinks, therefore, accounting for the fluctuations in the amount of soluble protein detected by the Lowry test at the different durations of microwave irradiation. However, the standard deviation of the triplicates of the protein controls was large overall, suggesting that lysozyme alone when being subjected to microwave irradiation underwent more conformational changes than when glucose was present.

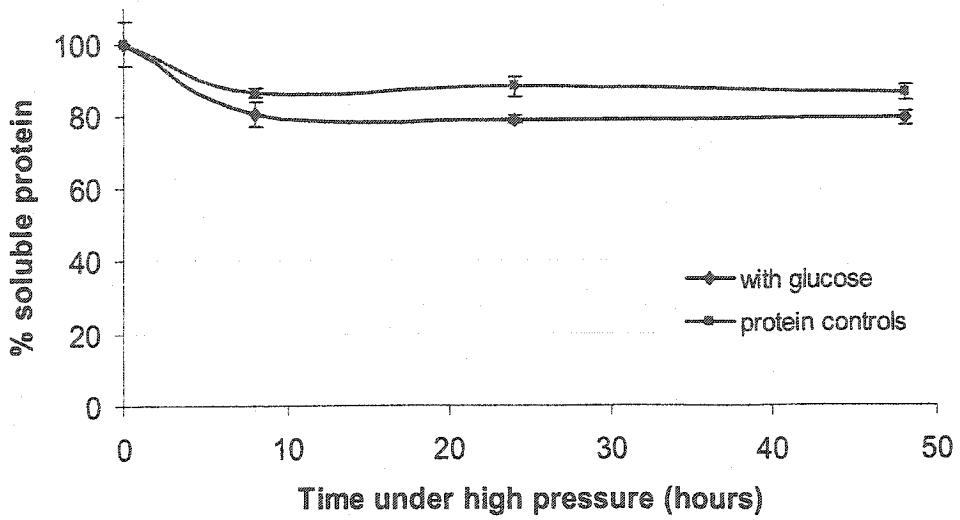


Figure 5.3 Percentage of soluble lysozyme remained in samples upon being subjected to 400 MPa of HHP from 8 hours to 48 hours at 30°C, as compared to the initial amount of protein at day 0.

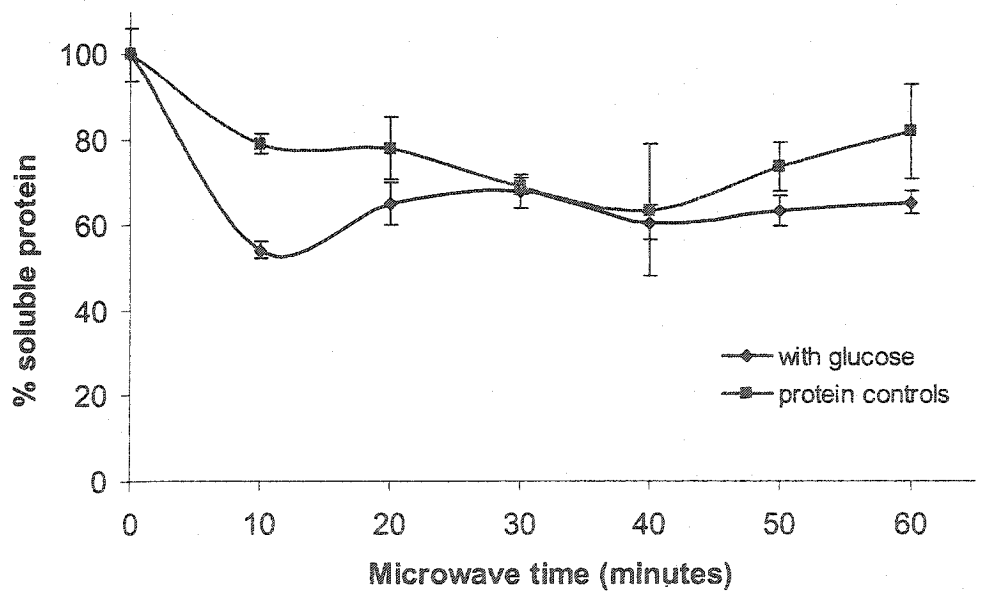


Figure 5.4 Percentage of soluble lysozyme remaining in samples subjected to microwave irradiation from 10 minutes to 60 minutes at 50°C, as compared to the initial amount of protein at day 0.

Out of the three treatments, high pressure at 30°C seems to be the process that causes the least damage to lysozyme, with 80% of its structural integrity protected upon processing. Microwave irradiation at 50°C comes next, and regular incubation at 30°C without the presence of antimicrobial agent such as sodium azide causes the most damage to the protein, up to some 55% of the protein was lost.

5.1.2 FLUORESCAMINE ASSAY

Lysine, being one of the most reactive amino acids in protein, reacts readily with glucose to generate glycated proteins. Assaying the amount of lysine that remains in the solution mixture by fluorescamine assay is therefore a way to identify the amount of intact ARP present. Figure 5.5a shows a non-linear (polynomial) correlation between the extent of glycation of the lysozyme in the lysozyme-glucose mixture that had been incubated at 30°C. There is a linear increase in the extent of glycation as a function of incubation time reaching around 20 to 25% glycation The extent of glycation increased as the incubation time increased, and this agrees with a study on egg albumin with glucose (Tanaka *et al.*, 1977) which found around 20% glycation of egg albumin upon 10 days of incubation at 37°C. As shown in Figure 5.5a, upon 6 days of incubation, the extent of glycation has reached its maximum capacity at 30°C, with most of the exposed lysyl groups reacted. The polynomial correlation also suggested that further incubation would induce loss of glycated proteins, either due to reversible reactions of ARP or crosslinks occurring within the protein. Average number of reacted lysyl groups in the samples calculated was only around 1.3 lysyl groups (See Table 4.7 for calculated results and Appendix A3.2 to A3.4 for steps in calculating the extent of glycation), and the average

number of reacted lysyl groups did not increase significantly after 6 days of incubation, and this agrees with a previous study on glycation of lysozyme (Yeboah *et al.*, 2000). The difference in the average number of reacted lysyl groups can be accounted by the differences in the temperature of the two experiments. Since temperature has been shown as a significant factor in promoting Maillard reaction, therefore, it is not surprising that with a lower temperature, the extent of glycation is comparatively lesser than at a higher temperature.

The presence of antimicrobial agents also affects the extent of glycation. The correlation between the extent of glycation of lysozyme-glucose mixture that contained sodium azide and the duration of incubation showed an overall linear increase (Figure 5.5b). The presence of 0.01% sodium azide served to destroy the micro-organisms or bacteria that were present in the system. The linearity of the correlation between the extent of glycation suggested that glycation occurred at an increasing pace but very slow. The lower extent of glycation as compared to that of the samples without sodium azide also suggested that minimal conformational change occurred with the presence of sodium azide, allowing only the lysyl groups that were exposed in the native state of lysozyme to react with glucose.

The extent of glycation of the high-pressure treated samples is shown in Figure 5.6. The optimum glycation was reached after 8 hours of pressurization. This optimum level accounted for 60% lysyl groups being glycated, which is about 3 times as much as subjecting the lysozyme-glucose mixture to 6 days of incubation. It has been reported that

high pressure accelerates the formation of the initial condensation product at the early stage of Maillard reaction - the Amadori stage (Tamaoka *et al.*, 1991; Issacs and Coulson, 1996; Bristow and Issacs, 1999). After 8 hours, the extent of glycation of lysozyme decreased. This may be due to the reversibility of the Schiff base or Amadori rearrangement, releasing the primary amino groups to be detected by the fluorescamine assay (Yaylayan and Huyghues-Despointes, 1996; Davidek *et al.*, in press). Another possible mechanism could be the presence of minute amounts of oxygen in the sealed plastic bags containing the samples that could allow for the browning reactions (Issacs and Coulson, 1996) and the formation of deoxyglucosones upon degradation of the Amadori compound with the release of the free amino groups (Zhao, 2001) to be able to react with fluorescamine, therefore, resulting in lower extent of glycation. A correlation between the extent of glycation and the duration of pressurization was not possible since the optimum point of glycation occurred at the first measured data point. Future work with more data points before 8 hours should be collected, so as to generate a correlation between the extent of glycation with the duration of pressurization.

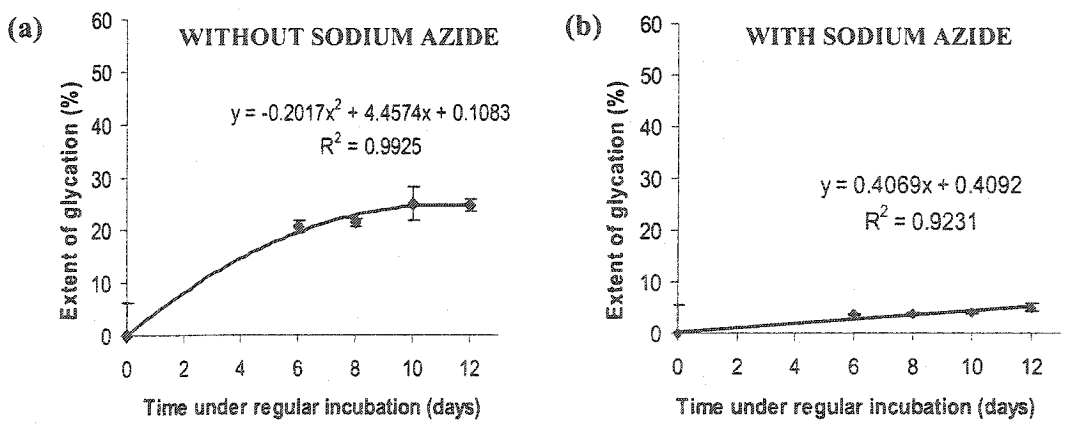


Figure 5.5 Correlation of the extent of glycation of lysozyme-glucose mixture that have been subjected to regular incubation at 30°C over a period of 12 days when (a) 0.01% sodium azide was absent; and (b) 0.01% sodium azide was present. Both are compared to the initial extent of glycation of the day 0 sample.

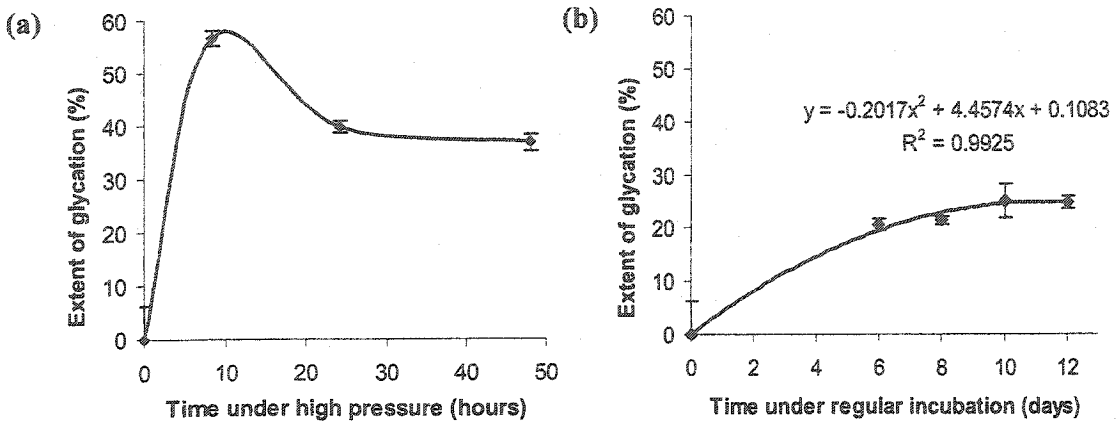


Figure 5.6 Extent of glycation of lysozyme-glucose mixture that have been subjected to (a) 400 MPa of HHP at 30°C over a period of 48 hours; and (b) regular incubation at 30°C over a period of 12 days, both are compared to the initial extent of glycation of the day 0 sample.

The extent of glycation of microwave-treated samples, over a period of 60 minutes at 50°C, varied between 30 to 43% with the maximum glycation reached after 30 min as shown in Figure 5.7a. This variation suggests that reversible reactions took place upon reaching a certain maximum extent of glycation. The glycated lysozyme seemed to undergo reversible reactions as observed in the samples that were being subjected to HHP, allowing for more free amino groups that got released to react with fluorescamine. It might possible that conformational changes due to folding and unfolding of the proteins took place throughout the 60-minute microwave irradiation period. The large standard deviation of the triplicates obtained for the 30-minute sample (±10.5%) suggested that the protein underwent random conformational changes, affecting the availability of lysine to react with glucose, and hence affecting the overall extent of glycation generated. Overall, glycation reached a maximum of around 40% with 20 minutes of microwave irradiation, which is comparable to two times the extent of glycation as when the lysozyme-glucose mixture was subjected to regular incubation at 30°C under standard conditions. To avoid

building up of excessive pressure during the microwave process, the centrifuge tubes containing the samples were not capped. As a result the samples might have experienced some moisture loss to the surrounding as the reaction progressed, which in turn decreases the availability of water as polarizable molecules for microwave absorption, as suggested by Yeo and Shibamato (1991). Therefore, there is a possibility that the free lysyl groups did not have sufficient energy to bind with the free glucose again upon the reversible reactions of ARP, resulting in a decrease in the extent of glycation as the treatment time increased.

The extent of glycation of lysozyme samples being incubated from 10 to 60 minutes at 50°C was also studied to eliminate the effect of temperature from microwave irradiation on glycation. Incubating the samples from 10 to 50 minutes did not show any sign of protein denaturation and glycation, while when the incubation time at 50°C increased to 60 minutes, there was a minimal 8% of glycation. In other words, the extent of glycation found on the microwave-treated samples can safely be assumed that it was the effect of microwave irradiation and not the temperature.

From the data obtained, there seems to be a non-linear (polynomial) correlation between the extent of glycation and the time of microwave irradiation (Figure 5.8). The polynomial correlation suggested that optimum glycation occurred at around 23 minutes under the experimental conditions, and the extent of glycation after this plateau would decrease upon prolonged exposure to microwave irradiation, suggesting the formation of irreversible crosslinks. Future work on validating the correlation between the extent of glycation and the exposure time of Amadori product to focused microwave irradiation

should be performed. In addition, the duration of microwave exposure should decrease to minutes to estimate the changes before 10 minutes, as to obtain a clearer picture in generating glycation with microwave irradiation.

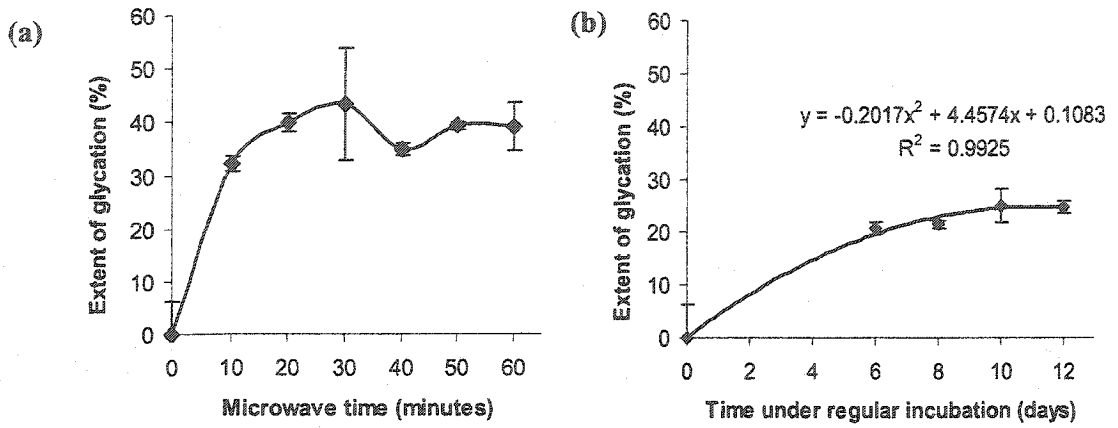


Figure 5.7 Extent of glycation of lysozyme-glucose mixture (a) subjected to focused microwave irradiation at 50°C over a period of 60 minutes; and (b) subjected to regular incubation at 30°C over a duration of 12 days, both are compared to the initial extent of glycation of the day 0 sample.

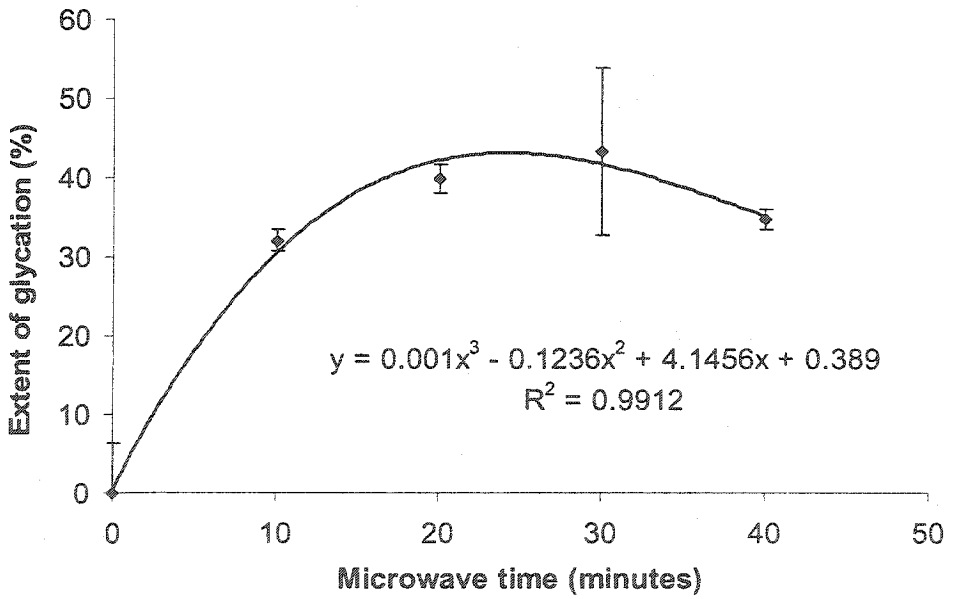


Figure 5.8 Correlation of the extent of glycation of lysozyme-glucose mixture versus the duration of microwave irradiation at 50°C.

5.1.3 COMPARISON OF THE EFFECT OF THE TWO TREATMENTS ON GLYCATION OF LYSOZYME

In comparing the different types of treatments, high pressure seems to cause the least protein destruction even at prolonged exposure time like 48 hours at 400 MPa, and at the same time achieving higher extent of glycation. For example, three times more glycation was observed in subjecting the samples to high pressure for 8 hours as compared to incubating the samples at 30°C for 6 days; or 2 times more glycation in 8 hours as compared to 10-minute of microwave irradiation. Microwave irradiation could generate around 30 to 40% glycation within 60 minutes. However, the protein loss was slightly higher for the microwave-treated samples. There was a non-linear (polynomial) correlation between the microwave time and the extent of glycation that can be achieved, with an optimum glycation of 43% at around 23 minutes, which was around 2 times as much as incubating the samples for 6 days at 30°C.

On the other hand, regular incubation at 30°C, causes the highest extent of protein destruction (around 50%) and only 20 to 25% of glycation could be achieved. However, addition of 0.01% sodium azide to the samples prevented damage or denaturation to the protein, but it did not seem to promote glycation.

5.2 EFFECT OF HHP AND MICROWAVE IRRADIATION ON BSA

BSA (66,000 Da) being a larger protein than lysozyme (14,300 Da) is more prone to degradation during thermal processing. It has been reported that when proteins and reducing sugars are heated, the corresponding solubility of the proteins would be reduced

along with occurrence of gelation. (Hill *et al.*, 1992; Yowell and Flurkey, 1986; Kaye *et al.*, 2001). Gelling occurred with BSA samples that had been subjected to HHP and focused microwave irradiation, in which, gelation was defined by Ferry (1948) is a two-step process with unfolding or dissociation of the protein molecules followed by association or aggregation reactions.

To carry out the tests to determine the protein content and the glycated lysyl groups, 12% SDS solutions were added to the gels and the subsequent soluble protein content was estimated from the Lowry test to calculate the extent of glycation.

5.2.1 LOWRY TEST

Samples of BSA that were incubated in the oven at 30°C are the only samples that did not gel, and remained in solution. The Lowry test, did not detect much protein loss for samples incubated with glucose; while the corresponding controls had approximately 10% loss in the protein content (Figure 5.9). The presence of glucose therefore seems to act as a protectant. The overall protein content of the BSA controls was lower than that of the BSA-glucose mixtures, and this further suggested the role of glucose in the mixture as a stabilizer to the protein. This observation also agrees well with a previous study in the reaction between human serum albumin and glucose (Brimer *et al.*, 1995). According to this study, the mild conditions of incubation or storage of food proteins in the presence of reducing sugars mainly affect lysine, thereby protecting the integrity of the other amino acids from crosslinking through lysyl residues (Hurrell and Carpenter, 1977). Therefore, due to Maillard reaction, the formation of isopeptides is retarded (Otterburn, 1989).

Isopeptide bond formation is one of the common types of crosslinks that can happen in proteins, especially in proteins that are rich in lysine since it can form a bond between its γ-amino group and an adjacent carboxylic acid (Lundblad, 1995). Furthermore, the standard deviations of the triplicates of the protein controls throughout the 12-day incubation were high, as compared to the glucose-containing samples. This further suggested that glucose acts as a stabilizer to the protein in *preventing* random conformational changes throughout the incubation period. The above observation seems in contradiction with a previous study in which, the percentage of soluble BSA of the BSA-glucose mixture incubated in the presence of oxygen experienced a gradual loss with time (Yeboah *et al.*, 1999). A possible explanation in the discrepancy between the two experiments is the presence of oxygen. Oxygen is an important factor in governing the protein loss since it allows for oxidation reactions. Samples in this study were placed in micro-centrifuge tubes with caps closed upon incubation, therefore securing minimal amounts of oxygen for degradation. In addition, differences on the physical state of the proteins upon incubation (dry versus solution) may also affect the rate of Maillard reaction since moisture content or water activity of the system is a critical factor that has an effect on many of the pathways of Maillard reaction and the extent of crosslinking (Wu *et al.*, 1990; Ames, 1998).

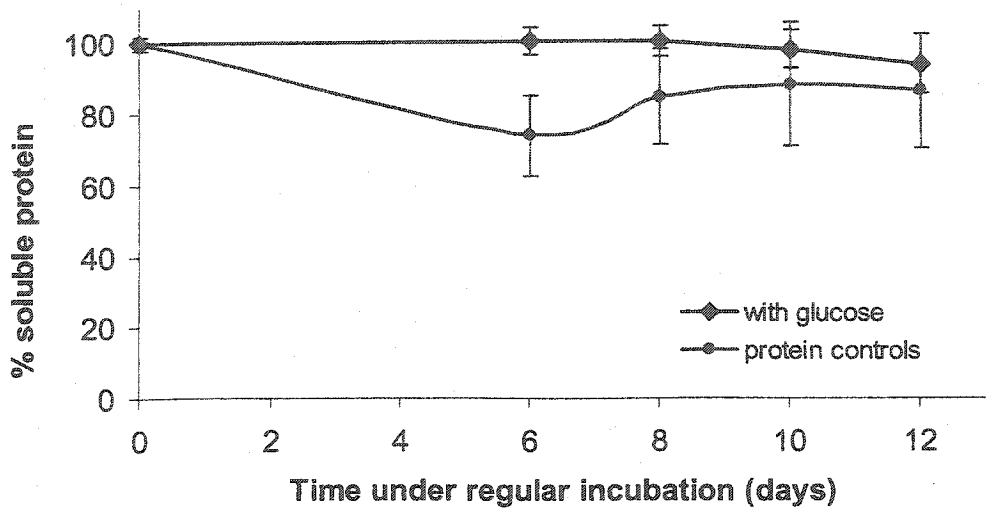


Figure 5.9 Percentage of water soluble BSA remained in the samples upon being subjected to regular incubation at 30°C over a period of 12 days, as compared to the initial amount of BSA at day 0.

Figure 5.10 shows the percentage of soluble BSA in the BSA-glucose mixtures and the BSA controls that have been subjected to HHP over a period of 48 hours. Significant losses in the protein content of around 90% and 70% for the controls and the glucose-containing samples respectively were observed over the 48 hours of incubation. Previous studies conducted on the denaturation of BSA under high pressure have found no signs of denaturation even at 400 MPa (Hayakawa *et al.*, 1992; Aoki *et al.*, 1966); similarly, when ovalbumin was subjected to pressures up to 800 MPa in the presence of sucrose also showed no signs of denaturation (Iametti *et al.*, 1998). While in the studies conducted by Galazka *et al.* (1997a,b) aggregation of BSA was observed upon pressurization. A possible explanation for the discrepancies between different studies could be attributed to the differences in the type of saccharides and in the concentration of the BSA solutions used since more concentrated solutions are more prone to decreased solubility upon HHP processing, leading to gelation. In addition, time of exposures to

pressure is also a factor in governing gelation, with a longer exposure time allowing the protein or protein mixture to gel and crosslink. In comparing the glucose-containing samples with the protein controls, the former seemed to be more capable of retaining the integrity of the protein due to the occurrence of Maillard reactions, resulting in higher amount of soluble protein after being processed. Nonetheless, as the processing time increased, more conformational changes occurred, leading to the occurrence of denaturation or irreversible crosslinks. It seems that the protein with the exposed side chains reacted with each other, inducing reversible crosslinks as in the samples that had been subjected to regular incubation. In addition, the standard deviation of the 8-hour pressurized sample that contained glucose was high, suggesting that the protein underwent significant conformational changes and random cross-linking. Protein controls, on the other hand, did not show randomized crosslinking with smaller standard deviations. Nonetheless, results showed that the protein content of both the glucose-containing BSA and the BSA alone reduced significantly upon subjection to 400 MPa of high pressure, despite the possible types of reversible crosslinks that could be formed. In addition, all the BSA samples gelled upon being subjected to high pressure. Gels were solubilized in SDS solutions for Lowry test and fluorescamine assay. The fact that SDS did not solubilize the gels effectively and efficiently upon vortexing and centrifuging, could also lead to underestimation of the protein content. Future work has to be done on more detailed amino acid analyses to detect the status of the amino acids in the sample. In addition, shorter HHP processed times such as minutes should be studied so as not to destroy the protein totally while trying to promote Maillard reaction.

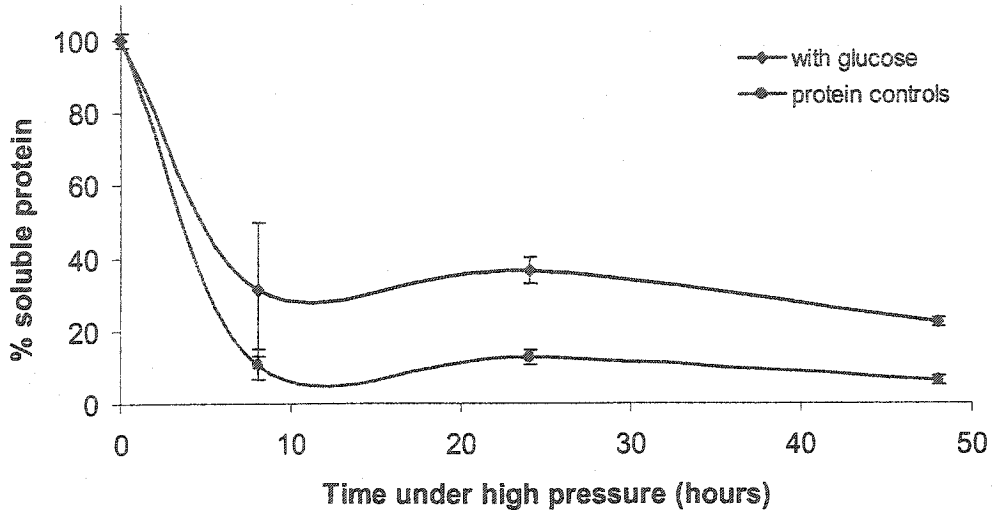


Figure 5.10 Percentage of soluble protein of BSA samples subjected to HHP at 30°C as compared to the initial amount of the protein at day 0.

Figure 5.11 showed the percentage of soluble protein content of the BSA samples that have been subjected to focused microwave irradiation over a period of 60 minutes. With the microwave treatment, the damage to the proteins was less, compared to those treated with high pressure. Microwave heating of proteins has been reported to have reduction in their water solubility (Yowell and Flurkey, 1986; Dowdie and Biede, 1983). Once again, the protein content of the glucose-containing samples was higher than that of the controls, further confirming the role of glucose as a stabilizer in protecting the proteins from further crosslinking with other amino acids of the protein during microwaving. However, the protein content of the BSA-glucose mixture kept decreasing, suggesting that a major amount of the protein got denatured irreversibly with loss in solubility upon prolonged exposure to microwave irradiation. At the same time, the protein content of the controls encountered almost total loss of the protein's solubility throughout the process of microwave irradiation, suggesting the occurrence of

irreversible crosslinks. Furthermore, the large standard deviations of the samples that contained glucose suggests that the conformational changes experienced by the protein were not as uniform as the changes that were experienced by the BSA in the absence of glucose.

Overall, BSA seems to be a protein not suitable for high energy exposures. High pressure treatment caused the greatest damage to BSA, followed by microwave irradiation, because as the BSA samples are being subjected to high energy exposures, competing reactions such as crosslinking of the proteins can take place more readily. Note that isopeptide bond formation is one of the common type of crosslinks that could occur in proteins that have many lysyl groups, and BSA is one of the proteins that contains numerous lysine groups (66 lysines), as well as glutamic and aspartic acids, which provides the carboxylic group for the lysine to crosslinking (Brown, 1975; Patterson and Geller, 1977; McGillivray *et al.*, 1979; Reed *et al.*, 1980; Hirayama *et al.*, 1990).

While for the samples that were being incubated at 30°C, the integrity of the protein was maintained, particularly for the samples that contained glucose upon incubation, confirming the role that glucose acts as a protectant in inhibiting crosslinking, while promoting Maillard reaction.

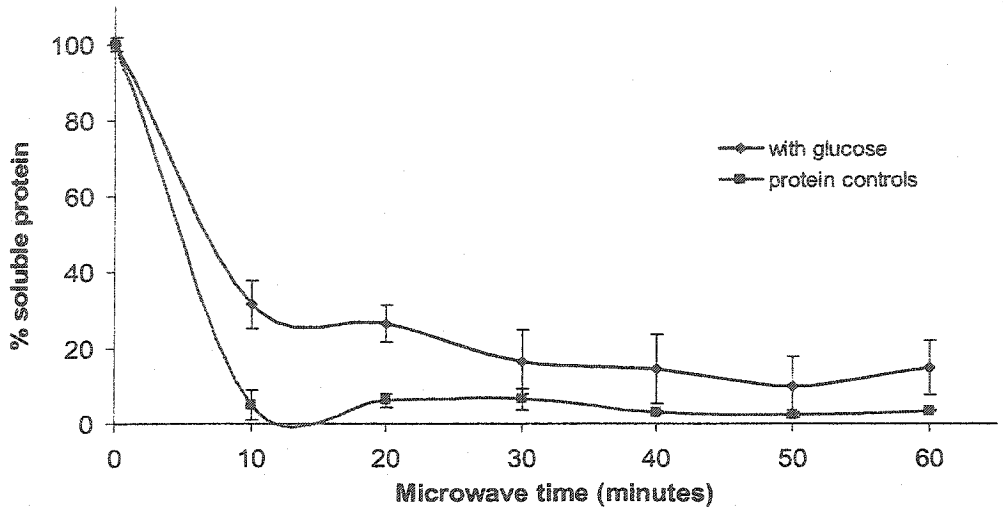


Figure 5.11 Percentage of soluble BSA in samples subjected to focused microwave irradiation from 10 minutes to 60 minutes at 50°C, as compared to the initial amount of the protein at day 0.

5.2.2 FLUORESCAMINE ASSAY

Figure 5.12 shows the correlation between the extent of glycation in the incubation samples of BSA for 6, 8, 10 and 12 days. Compared to the unmodified BSA-glucose sample at day 0, around 33% of the BSA got glycated on day 6, and the extent of glycation minimally increased up to 10 days of incubation, in agreement with a previous study on the reactivities of BSA (Yeboah *et al.*, 1999). Upon prolonged incubation, it seemed that the ARP underwent reversible changes in releasing the sugar moieties from the lysine residues. In addition, the non-linear (polynomial) correlation suggested that glycation reached a maximum at around 9 days with about 35% glycation which represents around 22 lysyl groups (out of a total of 61) (see Table 4.17 for calculated results and Appendix A3.2 to A3.4 for steps in calculating the extent of glycation).

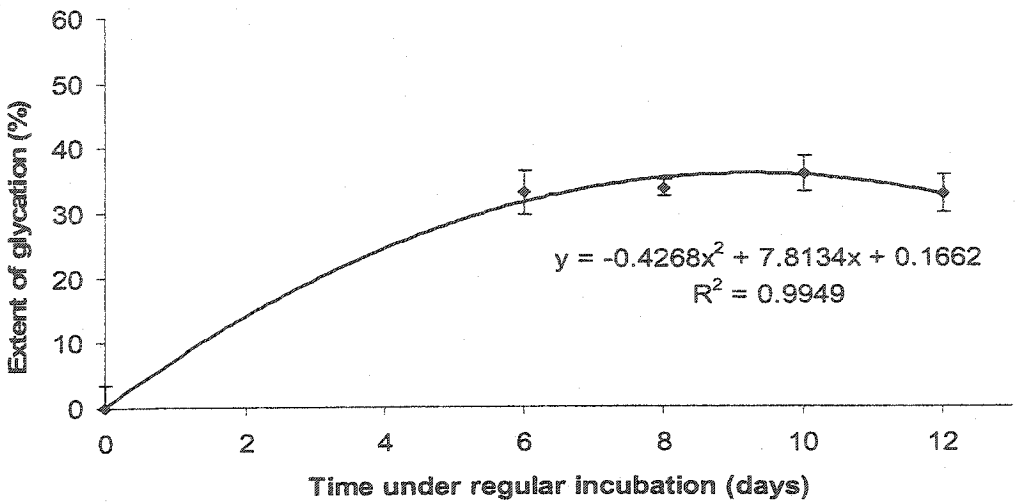


Figure 5.12 Correlation of the extent of glycation of BSA-glucose mixture versus time of incubation at 30°C relative to day 0 BSA-glucose sample.

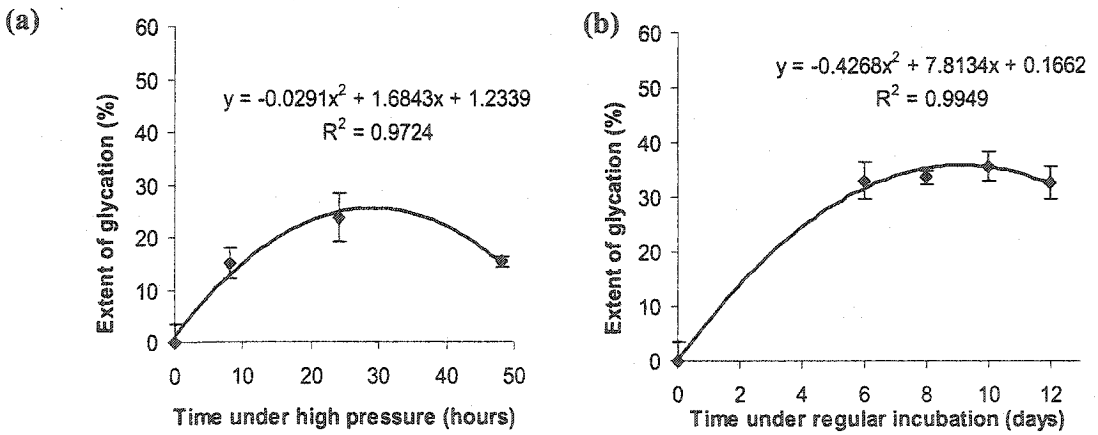


Figure 5.13 Correlation of the extent of glycation of BSA-glucose mixture versus the time of (a) HHP treatment at 30°C over a period of 48 hours; and (b) regular incubation at 30°C over a duration of 12 days, both are compared to the initial extent of glycation of the day 0 BSA-glucose sample.

When subjecting the BSA-glucose mixture to HHP over 48 hours, the correlation of the extent of glycation versus the time of the samples under pressure obtained is shown in Figure 5.13a. This figure indicates that with 8 hours of pressurization, BSA only attained some 12 to 15% of glycation, and the extent of glycation increased to around

24% for the 24-hour treatment, and reverted back to 15% for the 48-hour treatment. A possible explanation for the lower extent of glycation as compared to the incubation samples is that the glucose underwent rapid mutarotation from pyranose to furanose with the high pressure, leaving very small amounts of the free aldehydes which serves as intermediate for the conversion in the solution (Andersen and Gronlund, 1979; Andersen *et al.*, 1984), in which, the free aldehyde of glucose is the form that the lysine reacts with upon glycation. It seems that the rapid mutarotation slowed down the reaction between glucose and the lysine groups, and crosslinking of the proteins occurred at a faster rate than the lysine groups reacting with the aldehyde groups of the glucose.

Figure 5.14a shows the extent of glycation of the BSA-glucose mixture upon microwave irradiation at 50°C over a period of 60 minutes. The extent of glycation decreased after reaching a maximum at 10 minutes of irradiation. At this time 30% of the soluble BSA remained, suggesting that 70% of the protein were denatured. Fluorescamine assay estimated 17% glycation, representing an average number of 10 glycated lysyl groups. While on the other hand, regular incubation had around 100% of soluble protein upon 6 days of incubation, while attaining around 30% of glycation. This suggested that microwave irradiation provided an accelerating effect on both the Maillard reaction and protein denaturation as compared to the samples being subjected to regular incubation, and this is in agreement with the observation of Villamiel *et al.* (1996).

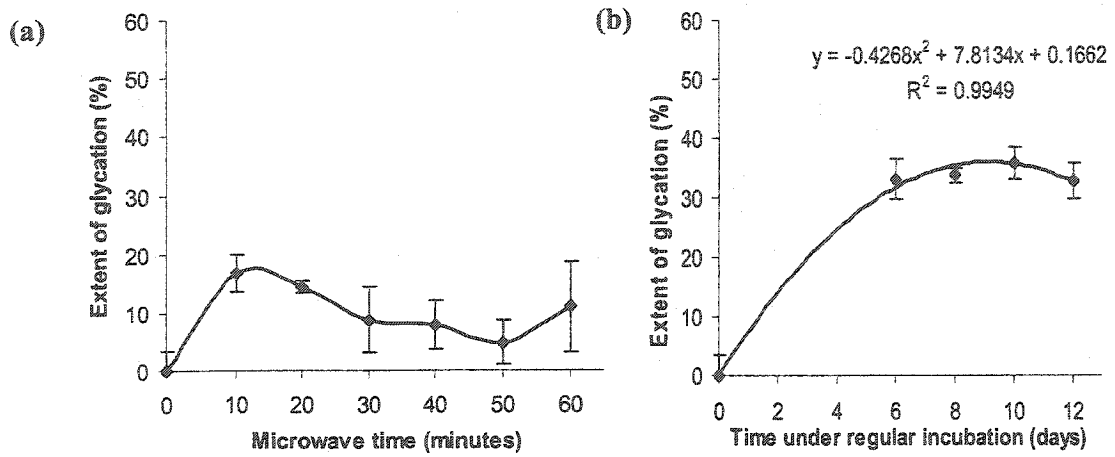


Figure 5.14 Extent of glycation of BSA-glucose mixtures (a) subjected to microwave irradiation at 50°C over a period of 60 minutes; and (b) subjected to regular incubation at 30°C over a period of 12 days, both are compared to the initial extent of glycation of the day 0 BSA-glucose sample.

The extent of glycation observed under microwave irradiation decreased as the microwave time increased, and followed the same trend as the high pressure treated samples between 24 and 48 hours of treatment. This observation suggests that the protein denaturation happens concurrently as the Maillard reaction. However, since the processing time with microwave irradiation is comparatively shorter than that of the high pressure treatment, denaturation was not as extensive as the HHP treated samples and more of the lysine groups in the proteins were free from being crosslinked and could react with the glucose moieties. The standard deviation of the 60-minute sample is comparatively higher, suggesting the occurrence of random conformational changes of the protein. The non-linear (polynomial) correlation between the extent of glycation and the microwave time shown in Figure 5.15 suggested that optimum glycation can be obtained with short durations of microwave exposure of around 20 minutes, and prolonged exposure would only result in denaturation of the protein.

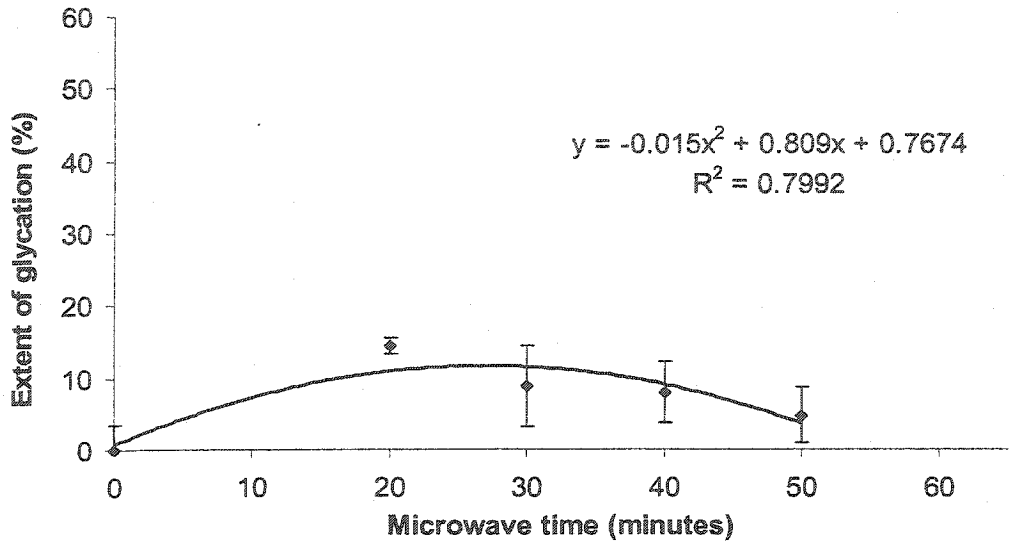


Figure 5.15 Correlation of the extent of glycation of BSA-glucose mixture versus the time under microwave irradiation.

5.2.3 COMPARISON OF THE EFFECT OF THE TWO TREATMENTS ON GLYCATION OF BSA

Comparison of the effect of different types of treatments on BSA indicates that BSA is very susceptible to crosslinking or other mechanisms that may damage the proteins. The results also indicate that high energy exposure regardless of duration severely damages the protein. An 8-hour HHP treatment at 400 MPa could only attain one fourth the extent of glycation to that of 6-day regular incubation at 30°C; at the same time, a 10-minute microwave irradiation could only attain half of the extent of glycation to that of a 6-day regular incubation, furthermore, a 10-minute of microwave irradiation attained comparable extent of glycation to that of 8-hour HHP treatment. Future work should be done on obtaining optimum extent of glycation with the least damage to the proteins with shorter durations of high energy exposures.

5.3 OVERALL COMPARISON OF THE EFFECT OF DIFFERENT TREATMENTS ON BSA AND LYSOZYME

In comparing the effect of the two high energy exposures on the two proteins, it can be concluded that lysozyme is the protein that will not undergo much degradation upon prolonged high energy treatments with appreciable extent of glycation from 30 to 60%. With around 80% of soluble protein remaining after HHP treatment, extent of glycation of the soluble protein could reach up to 60%. For the microwave-treated samples, 60% of the lysozyme remained active upon processing attaining 40% glycation. Incubating the lysozyme-glucose solution at 30°C over a period of 12 days encountered the most damage to the protein, leaving only about 40% of the protein active, and generated about 20% of glycation. On the other hand, BSA was very susceptible to high energy exposure and experienced high protein loss. BSA-glucose solution experienced around 70 to 80% protein loss upon treatment with HHP and around 80 to 90% with microwave irradiation. The protein content of the microwave-irradiated samples was shown to decrease as the microwave time increased. However, the protein content of the BSA-glucose samples experienced negligible loss upon incubation for up to 12 days at 30°C. The extent of glycation was also negligible for the samples treated with high energy exposures, with only 10 to 20% of the soluble protein achieving glycation. On the other hand, while glucose did not protect the integrity of lysozyme, however it did so to BSA. Less BSA got denatured upon high energy exposure compared to the controls with no glucose added. The addition of sodium azide served to protect lysozyme from microbial degradation during incubation, nonetheless, it did not seem to promote glycation.

From a practical point of view, high energy exposure is not suitable for generating glycated BSA. Even with a 100% soluble protein remaining under regular incubation, only 35% was glycated. This suggests that a large protein with many lysyl groups is hard to glycate, because the lysyl groups remain hidden within the protein matrix and are not exposed to the environment for glycation. On the other hand, lysozyme, being a comparatively smaller protein with only 14,300 Da and 6 lysyl groups, it can more readily undergo conformational changes to expose the lysyl groups for glycation. Subjecting the lysozyme-glucose mixture to high energy exposures can actually accelerate the reaction between glucose and the lysyl groups, increasing the extent of glycation.

5.4 FUTURE STUDIES

To generate a better picture of glycation of proteins, HHP processing and microwave irradiation experiments could be performed at shorter reaction times so as to obtain information on the initial progress of glycation. In addition, operating parameters such as the magnitude of HHP applied, temperature of the microwave and HHP reactions, should be investigated so as to better promote Maillard reaction effectively in a relatively short period of time. More detailed analyses such as amino acid analysis could be done to reveal the actual amino acid content of the proteins upon being treated, with the extent of glycation to be estimated more precisely. Spectroscopic studies of the treated proteins should be carried out to validate the changes in the conformation of the proteins upon the different types of treatments.

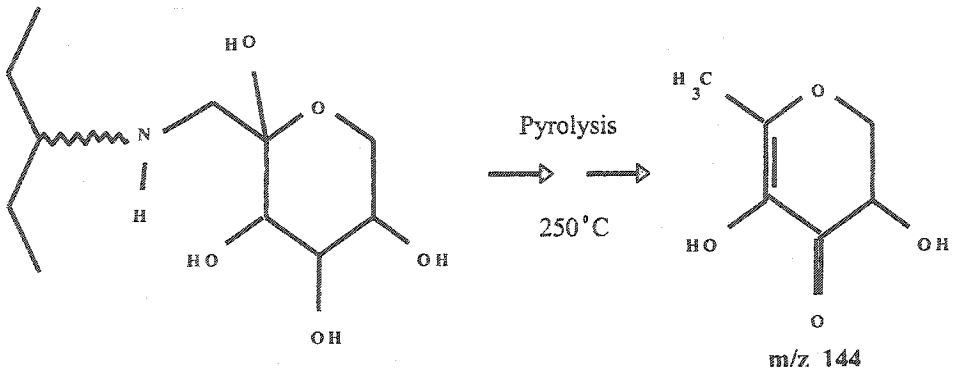
6 ANALYSIS OF GLYCATED PROTEINS BY PYROLYSIS-GC/MS

6.1 INTRODUCTION

There are numerous methods available for detecting the presence of glycated proteins. Methods such as TBA method, periodate oxidation, borohydride reduction, NBT colorimetric assay, ferricyanide test, Elson-Morgan test, etc. are some of the known methods for detection of Amadori product or glycated proteins. Separation-based assays using chromatography can be used to separate glycated proteins from unglycated species based on the charges of the species involved. Glycated proteins can also undergo hydrolysis and form furosine which can be separated by HPLC and then detected by measuring the corresponding absorbance at 280 nm (Furth, 1988). Fluorescence generated from the reaction of lysine with fluorescamine can as well be measured at 390 and 475 nm (emission and excitation wavelengths) to assay the extent of glycation (Yaylayan *et al.*, 1992). Other methods such as using infrared spectroscopy to detect and quantitate the open chain or keto forms of Amadori products, and subsequently used to infer the amount of Amadori product present, is a comparatively fast technique, however, lack of sensitivity may hinder its use as a practical method (Yaylayan and Huyghues-Despointes, 1994). Other spectroscopic methods such as GC/MS (gas chromatography – mass spectroscopy) are available for detection of glycated proteins, that require the samples to be derivatized or hydrolyzed prior to analysis, so that the compounds can be injected into the column to be analyzed. Common mass spectroscopic methods used nowadays such as ESI-MS (Electrospray ionization mass spectroscopy) and MALDI-MS (matrix assisted laser induced ionization mass spectroscopy) require less sample pretreatment steps as compared to regular GC/MS (Yeboah and Yaylayan, 2001). With

the use of mass spectroscopic methods, the intact glycated proteins can be analyzed qualitatively and quantitatively. Changes in the conformation of the proteins, the glycation sites and the distribution profile of glycoforms upon glycation of the proteins are some of the qualitative aspects that can be monitored by ESI-MS (Yeboah *et al.*, 2000).

All the above methods can be used to detect glycation in proteins, nonetheless, drawbacks for the chemical methods include susceptibility to interference by small-molecule contaminants or protein-bound groups in unpurified preparations, as well as enzymatically bound sugars, affecting the true readings of the test (Furth, 1988). The time consuming sample preparation steps for the spectroscopic methods such as derivatization or hydrolysis prior to analysis make them inconvenient to be used. Pyrolysis-GC/MS is therefore an alternative method that can be considered to detect protein glycation, since it is ideally suited to handle non-volatile compounds without tedious sample preparation. This proposition is based on the fact that any hexose moiety, whether free or existing as ARP such as in glycated proteins, can be decomposed to produce the characteristic 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one peak (M^+ at m/z 144) upon pyrolysis (Figure 6.1) at temperatures higher than 100°C (Yaylayan and Keyhani, 1996).



Glycated protein

Diagnostic ion for the presence of Amadori compound on proteins

Figure 6.1 The degradation of Amadori rearrangement product upon pyrolysis with the production of the ion at m/z 144.

The breakdown of a complex structure into smaller units for identification has been regularly used as an analytical technique (Mackillop, 1968). Pyrolysis is a technique that involves the breakdown of large complex molecules into smaller and more analytically useful fragments by applying direct heat. Pyrolysis is able to generate fragments in a reproducible manner when the heat energy that is applied to the molecule is greater than the energy of its specific bonds. Therefore, the bonds will dissociate in a predictable and reproducible way for analysis (Wampler and Levy, 1987). Coupling the pyrolyzer with analytical tools such as GC/MS or FTIR spectroscopy serves to interprete the identity of these fragments and to understand the structure of the original macromolecule. Compared to conventional methods of lengthy extractions or derivatization in analyzing insoluble materials such as wood, rubber, plastic, etc. by GC, MS and FTIR, pyrolysis is a much faster technique that requires small amount of samples with minimum sample preparation for reproducible results qualitatively and quantitatively (Goodacre and Kell, 1996; Yaylayan and Keyhani, 1996). Pyrolysis-

GC/MS methodology therefore, would serve as a rapid and accurate technique of detecting the amount of intact ARP present using the M^+ ion at m/z 144 as a diagnostic marker. The objective of this part of the study is to investigate the use of the M^+ ion at m/z 144 as a universal marker for glycated proteins by Pyrolysis-GC/MS assay, and to generate a subsequent correlation in glycation with the duration of incubation.

6.2 MATERIALS AND METHODS

6.2.1 MATERIALS

Glycated human albumin (GHSA, 95 % protein, 2.7 mol hexose/mol), human albumin (HSA), lysozyme, D-glucose and silica gel obtained from Sigma Chemicals (St. Louis, MO., USA), glycated lysozymes (LG-2, LG-5, LG-10 and LG-14) were generated according to the method of Yeboah *et al.* (2000). Lysozyme samples treated with 8 hours of HHP and 10 minutes of microwave irradiation (see Section 3.5 and 3.6.1) were freeze-dried for further use in this study. The quartz tubes with 0.3 mm thickness used were obtained from CDS Analytical Inc. (Oxford, OA). A Varian Saturn 2000 GC/MS interfaced to a CDS Pyroprobe 2000 unit (CDS Analytical Inc., Oxford, PA) with a fused silica DB-5 MS column (50 m length x 0.2 mm i.d. x 0.33 μm film thickness; J&W Scientific) was used for the Pyrolysis-GC/MS assay.

6.2.2 SAMPLE PREPARATION

6.2.2.1 INTERFERENCE OF FREE GLUCOSE WITH PYROLYSIS ASSAY

Glycated protein mixtures prepared from 8 hours of HHP and 10 minutes of focused microwave irradiation were freeze-dried for pyrolysis. Table 6.1 shows the

amount of protein, glycated protein, glucose and silica gel added to individual batches with thorough grinding. The two proteins used (lysozyme and HSA) were mixed with silica gel with or without glucose. One mg of solid sample from each batch was placed in a quartz tube for pyrolysis which contained 0.8 mg of the protein involved. In addition, glucose (1 mg) was pyrolyzed separately. Samples were introduced into quartz tubes plugged with quartz wool and pyrolyzed.

6.2.2.2 CORRELATION OF THE INTENSITY OF THE PYROLYSIS-GENERATED MARKER PEAK FROM COMMERCIALLY AVAILABLE GLYCATED HUMAN SERUM ALBUMIN (GHSA) WITH THE AMOUNT OF PROTEIN

Seven mixtures of commercial GHSA and silica gel were prepared with thorough grinding, with increasing concentrations of GHSA in each batch (i.e. 1 or 2 mg of the solid mixture from each batch contained GHSA ranging from 0.1918 mg to 1.4074 mg, with the remaining amount made up by silica gel). Two replicate pyrolysis were performed from each batch, and the 7 concentrations of the mixtures were tabulated in Table 6.2. Samples introduced into quartz tubes were plugged with quartz wool and pyrolyzed.

6.2.2.3 CORRELATION OF THE INTENSITY OF PYROLYSIS-GENERATED MARKER PEAK FROM PREPARED GLYCATED LYSOZYME WITH THE DEGREE OF GLYCATION

LG-2, LG-5, LG-10 and LG-14 were glycated lysozyme generated by incubating lysozyme at 50°C with glucose for 2, 5, 10 and 14 days respectively according to the method of Yeboah *et al.* (2000), followed by dialysis to remove unreacted sugars and freeze drying. Each of the samples above (3.2 mg) was mixed thoroughly with silica gel

(0.8 mg) and the mixture (1 mg) was introduced into quartz tube plugged with quartz wool for pyrolysis.

Table 6.1 The constituent of the samples duly prepared for pyrolysis. Lysozyme sample treated with 8 hours of HHP is designated by HHP, and sample treated with 10 minutes of microwave irradiation is designated by MW.

Batch name	Weight of protein involved (mg)	Weight of glucose (mg)	Weight of silica gel (mg)
HSA	3.2	-	0.8
HSA + glucose	3.2	0.3	0.5
GHSA	1.6	-	0.4
Lysozyme	3.2	-	0.8
Lysozyme + glucose	3.2	0.3	0.5
Glucose	1.0	-	1.0
HHP	0.8	-	0.2
MW	0.8	-	0.2

Table 6.2 The weight of GHSA and silica gel in the mixture prepared for pyrolysis.

Batch #	Weight of GHSA in mixture (g)	Weight of silica gel in mixture (g)	Amount of mixture pyrolyzed (mg)
1	0.0014	0.0059	1.0
2	0.0012	0.0021	1.0
3	0.0019	0.0028	1.0
4	0.0026	0.0016	1.0
5	0.0035	0.0009	1.0
6	0.0032	0.0021	2.0
7	0.0038	0.0016	2.0

6.2.3 METHODS

In all pyrolysis experiments, the pyroprobe interface temperature was set at 250°C at a heating rate of 50°C ms^{-1} with a total heating time of 20 sec. The mass range analyzed was 29 – 300 amu. The volatiles generated were sent to the sample preconcentration trap (SPT) at –30°C and trapped, followed by desorption at 250°C. The

volatiles were injected with a split ratio of 260:1, and were analyzed under delayed pulsed pressure. Pressure was increased from 1 psi to 70 psi at a rate of 400 psi min^{-1} and held for 2 min, then dropped to 2 psi at a rate of 400 psi min^{-1} to establish a constant flow of 1.5 mL min^{-1}. The initial temperature of the column was set at –5°C for 12 minutes, and then increased to 50°C at a rate of 50°C min^{-1}; the temperature was then further increased to 250°C at a rate of 8°C min^{-1} and kept at 250°C for 5 min. The ions formed from the ARP were identified and quantified with the relative intensity through library search of NIST and SATURN libraries of the SATURN software. Automated Mass spectra Deconvolution and Identification System (AMDIS), Version 2.0 – DTRA/NIST 1999) is the program that was used for deconvolution of components detected from Pyrolysis-GC/MS. Curve fitting equations were generated by Microsoft® Excel (2002) software package.

6.3 RESULTS AND DISCUSSION

6.3.1 INTERFERENCE OF FREE GLUCOSE WITH PYROLYSIS ASSAY

Pyrogram of pure glucose serves as a guide in confirming the retention time of the peak generated from 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (M^+ at m/z 144), which is present at around 26.20 min under the experimental conditions used (Peak denoted by I in Figure 6.2). In addition, the major peak detected in the pyrogram of pure glucose was hydroxymethylfurfural (HMF; peak denoted by H in Figure 6.2), which can be formed during dehydration or degradation of free hexoses (Kroh, 1994; Anam and Dart, 1995). Detection of HMF in the pyrolysis mixtures can therefore be used to indicate

the presence of free glucose, since Amadori products do not generate significant intensity of HMF (Yaylayan and Keyhani, 1996).

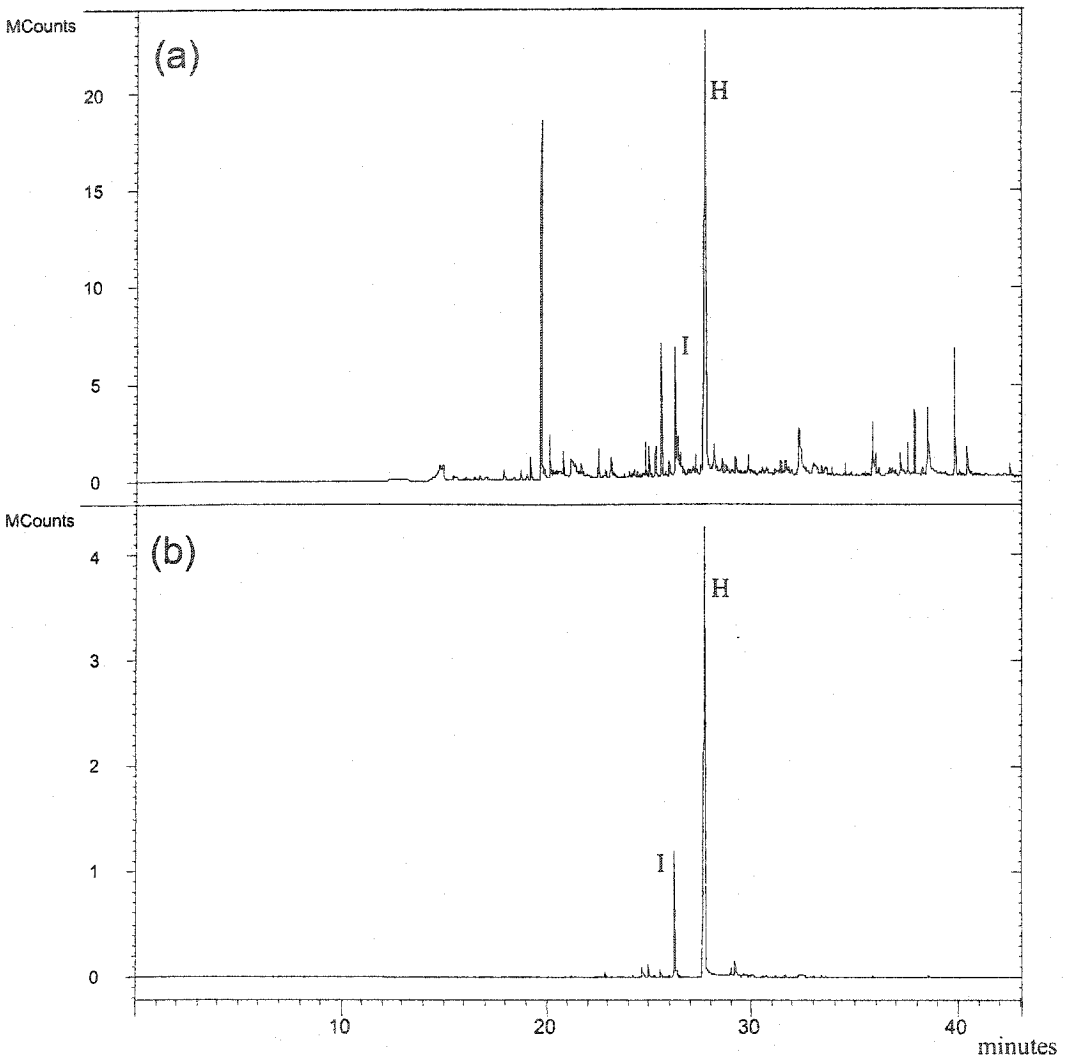


Figure 6.2 Pyrograms of glucose showing (a) total ion current and (b) Extracted Ion chromatograms (m/z 144 and m/z 126). Peaks containing the M^+ ion at m/z 144 and the ion from HMF at m/z 126 are designated by I and H respectively.

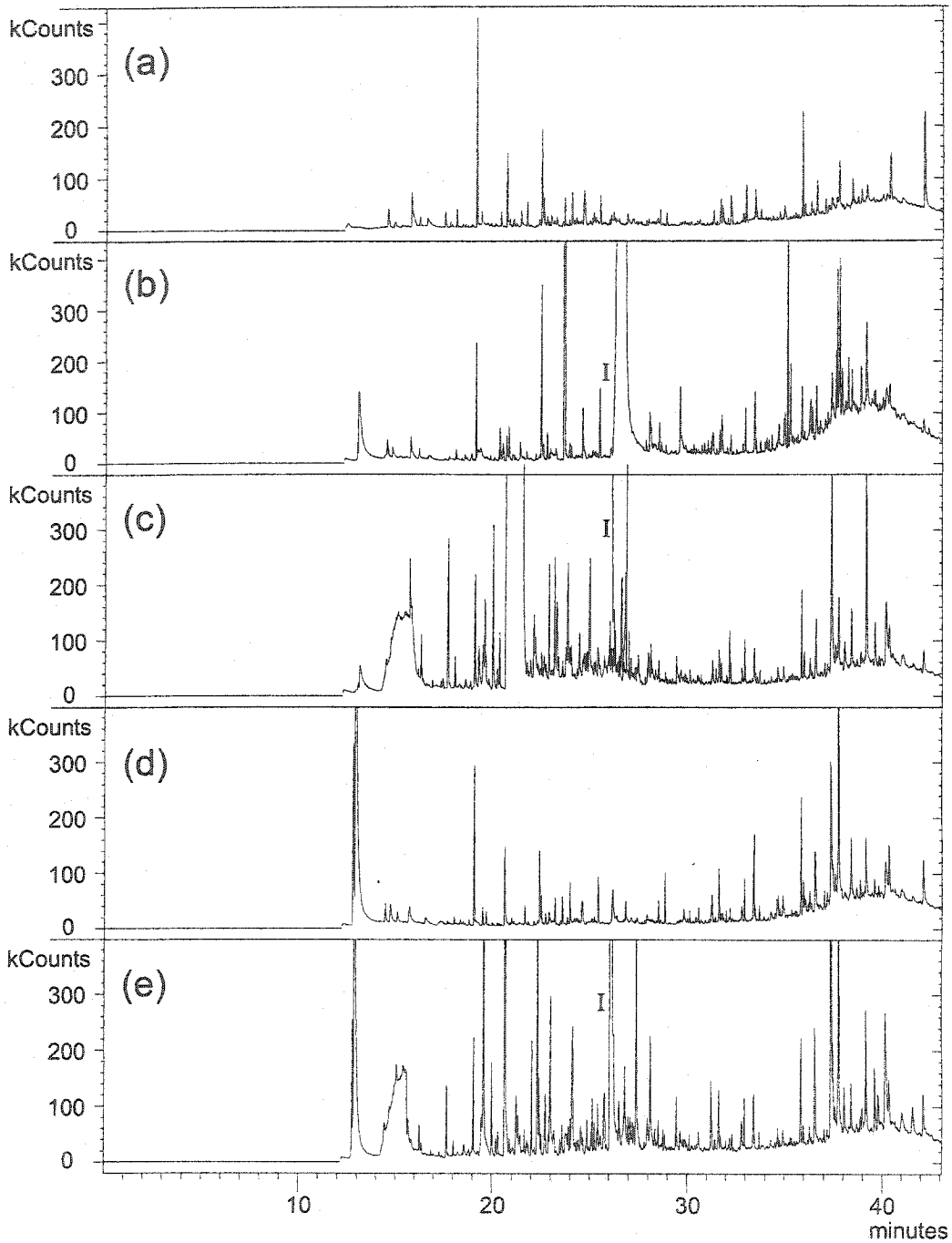


Figure 6.3 Pyrograms showing the total ion current of (a) HSA; (b) GHSA; (c) HSA with glucose; (d) lysozyme; (e) lysozyme with glucose. Peaks containing the M^+ ion at m/z 144 are designated by I in the pyrograms.

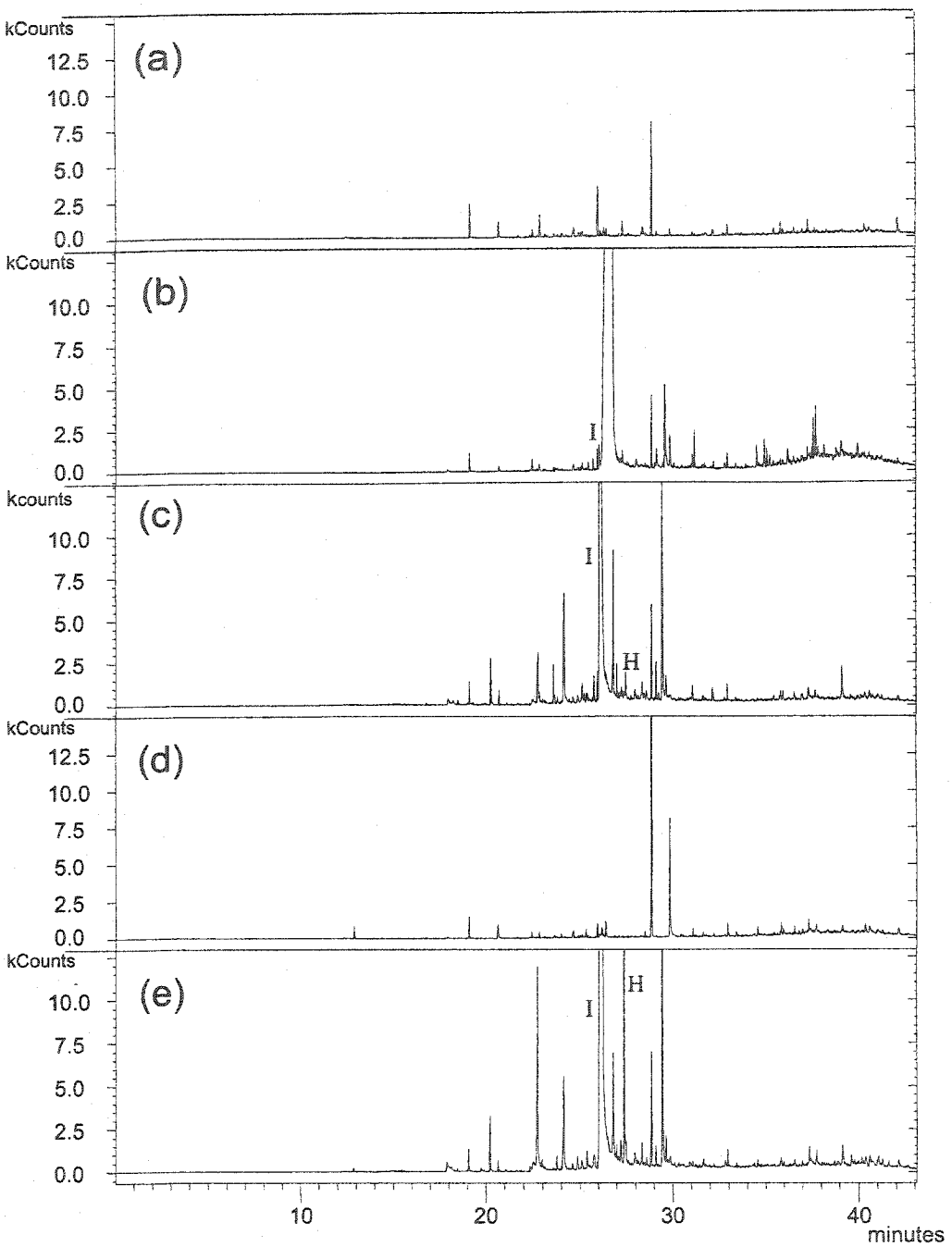


Figure 6.4 Extracted Ion chromatograms (m/z 144 and m/z 126) of (a) HSA; (b) GHSA; (c) HSA with glucose; (d) lysozyme; (e) lysozyme with glucose. Peaks containing the M^+ ion at m/z 144 and the ion from HMF at m/z 126 are designated by I and H respectively.

In order to assess the impact of free glucose on the pyrolysis assay, HSA, and HSA with addition of 10% (w/w) glucose were pyrolyzed (Figures 6.3a and c). Difference in intensities between glucose (1 mg) and HSA with glucose (1 mg containing both constituents) is due to the difference in the amounts of glucose in the two samples. The overall fragmentation pattern of HSA with glucose was a combination of fragments of HSA and glucose when they were pyrolyzed separately. On the other hand, glycated HSA gave a completely different pyrogram, generating different fragments from that of HSA and/or glucose upon being pyrolyzed (Figure 6.3b). Similar results were obtained when pure lysozyme and lysozyme with glucose were pyrolyzed (Figures 6.3d and e). This suggests that pyrolysis can be used as an analytical tool to distinguish proteins-sugar mixtures from glycated proteins. The corresponding Extracted Ion chromatograms for m/z 144 are shown in Figure 6.4. Note that glycated HSA, HSA with glucose, lysozyme with glucose and glucose alone all generated the M^+ ion fragment with m/z 144 from the decomposition of the sugar moieties having a retention time at exactly 26.29 min (±0.06 min). All sugar moieties generate the same fragment (M^+ ion at m/z 144) at the same retention time, suggesting that pyrolysis can be used as a fast analytical tool to distinguish glycated proteins from unglycated proteins and detect the presence of glycated proteins, provided that the samples have been properly dialyzed to remove any unreacted sugar and freeze-dried.

In addition, as shown in Figure 6.2, pyrolysis of glucose alone generated four fold excess of HMF relative to the glycation marker with M^+ at m/z 144. Pyrograms of HSA and lysozyme that contained glucose also contained HMF, but was absent in GHSA

(Figures 6.4b, c and d). This suggests that HMF could be used as an indicator for the presence of free glucose. However, further work is required to validate and correlate this assumption with the amount of glycated proteins, pure proteins and free glucose that could be present.

6.3.2 ESTABILSHING THE LIMIT OF DETECTION OF MARKER IONS FOR GLYCATED HUMAN SERUM ALBUMIN

In determining the limit of detection for detecting glycation of commercially available glycated protein, GHSA, the presence of the diagnostic marker compound 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (M^+ at m/z 144) was investigated. This ion was absent in pyrogram of pure HSA (Figure 6.5). Upon pyrolyzing 0.1918 mg to 1.4074 mg of GHSA, the marker compound only appeared in the highest concentration (Table 6.3). The corresponding signal intensities of the duplicates generated from deconvoluting the peak was 0.00697% and 0.00768% relative to the integrated TIC (total ion current) of the whole chromatogram, this correlates to the presence 5.76×10^{-8} moles of hexose based on 2.7 moles of hexose/mole of protein found in the starting GHSA. In other words, the limit of detection of GHSA was found to be 1.4074 mg. Future work on pyrolyzing amounts of GHSA slightly higher and lower than 1.4074 mg can be done so as to generate a calibration equation for GHSA.

6.3.3 PREDICTION OF THE EXTENT OF GLYCATION IN GLYCATED LYSOZYME WITH PYROLYSIS ASSAY

Figure 6.6 shows the TIC of the glycated lysozymes at different incubation periods. The overall degradation pattern of the lysozyme remained constant with minimal

difference as the incubation period increased. When only the diagnostic ion at m/z 144 was extracted from the pyrograms, the intensity of the peak at 26.29 min increased from 30 kCounts for 2-day incubation to more than 300 kCounts for 14-day incubation (Figure 6.7). Samples were dialyzed and freeze-dried to remove any unreacted sugar, so the intensity of the signal solely referred to the sugar moieties that were attached to the protein upon incubation. This suggests that increasing the incubation period increased the amount of sugar moieties attached to the protein, and this is in agreement with the increase in the average number of sugar molecules attached per molecule of lysozyme (Yeboah *et al.*, 2000). Quantitating the signal intensity of the deconvoluted peaks at retention time of 26.29 min, a linear correlation between the signal intensity at m/z 144 with the duration of incubation was obtained (Figure 6.8). In addition, when the signal intensity was correlated with the average sugar loading values of the lysozyme reported in literature (determined by ESI-MS analysis), a linear correlation was generated as well, which can be used to track and predict the average sugar loads of any glycated lysozyme prepared with the same procedure (Figure 6.9).

6.3.4 INTRODUCTION TO THE CONCEPT OF SIMILARITY INDEX TO ASSESS STRUCTURAL CHANGES IN PROCESSED PROTEINS

Lysozyme/glucose mixtures that had been subjected to 8 hours of HHP and 10 minutes of microwave irradiation were pyrolyzed after the freeze-drying step. By visual examination of the pyrograms generated, the two pyrograms seemed to show similar number of peaks with minimal difference in their intensities (Figure 6.10). Fluorescamine assay however, indicated that lysozyme sample subjected to 8 hours of HHP generated 2 times as much glycation as the sample that was subjected to 10 minutes of microwave

irradiation. To evaluate the differences between these two samples more accurately, their total ion chromatograms were compared with the total ion chromatogram of a model reference system containing lysozyme and glucose. Spectral comparison parameters - %Purity and %Fit values - were generated by the SATURN software and listed in Table 6.4. "%Purity" refers to the difference in the number of peaks generated between the tested sample and the reference, and "%Fit" refers to how similar the same peaks that are common in both the reference and the tested sample are in terms of their intensity. Since both parameters are equally important to assess the differences between the two samples, we introduced the concept of "Similarity Index" (SI) by adding the two values and dividing by 2, the obtained values for different systems are listed in Table 6.4.

Inspection of Table 6.4 indicates that when the reference system is lysozyme/glucose (R1), the similarity index (SI) of the microwave treated sample (SI=88.2) is higher than that of HHP treated sample (SI=76.5). This is consistent with the glycation values with the HHP sample having more glycation determined with the fluorescamine assay. A high similarity index to that of lysozyme/glucose reference system indicates presence of more unreacted protein and sugar in the test sample and a lower SI value indicates the presence of less free sugar and protein, and more glycated lysozyme is in the test sample. As expected, the SI values for pure lysozyme (SI=62.6), pure glycated lysozyme LG-2 (SI=59.1) and pure glucose (SI=62.4) were much lower as shown in Table 6.4, due to the difference in structures of the samples. It is expected therefore when the reference system is changed into glycated lysozyme (R2), the SI values will change such that the sample with higher glycation (HHP treated) will be more

similar to the reference since it had more glycation as determined by the fluorescamine assay. Inspection of the values reported in Table 6.4 confirms this prediction. In addition, the similarity indices reported in Table 6.4 for LG-5, LG-10 and LG-14 glycated samples relative to LG-2 can be used to predict the differences or similarities in their relative functional properties.

6.4 CONCLUSION

The feasibility of using Pyrolysis-GC/MS as an analytical technique to detect glycation was tested. Free glucose in the reaction system and bound glucose as ARP generated different markers upon pyrolysis, which can be used as a tool to distinguish unglycated proteins from glycated proteins. Attempt to generate a universal calibration based on the intensity of the M^+ ion at m/z 144 for glycated proteins was not possible, since different proteins have different efficiencies to generate the marker due to differences in their size and glycation sites. Generation of a calibration for glycation therefore is protein specific. The limit of detection of the diagnostic ion at m/z 144 for GHSA which has 2.7 moles of hexose per mole of protein and a 66,000 Da molecular weight was found to be 1.4074 mg. Lysozyme samples with different degrees of glycation produced a linear response with the signal intensity of the diagnostic ion at m/z 144. In addition, the subsequent sugar loading values of the glycated lysozyme also correlated linearly with the signal intensity produced by the marker. Finally, the introduction of the concept of similarity index based on total ion current of pyrograms can help to predict the changes in the structures of the glycated proteins and hence the subsequent functional properties.

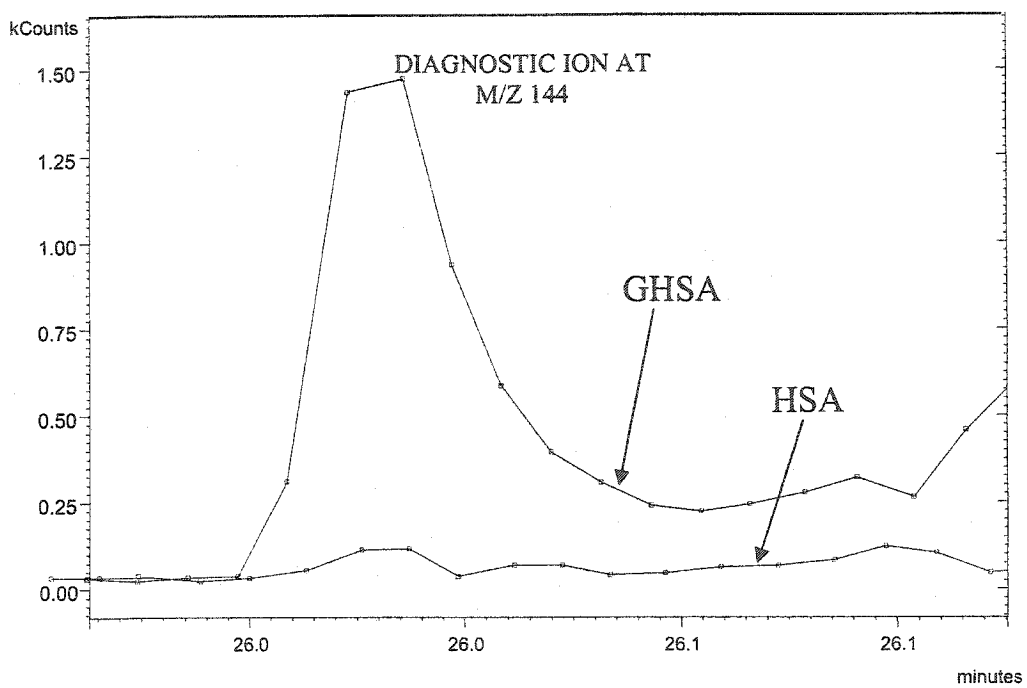


Figure 6.5 Overlaid chromatograms of HSA and GHSA.

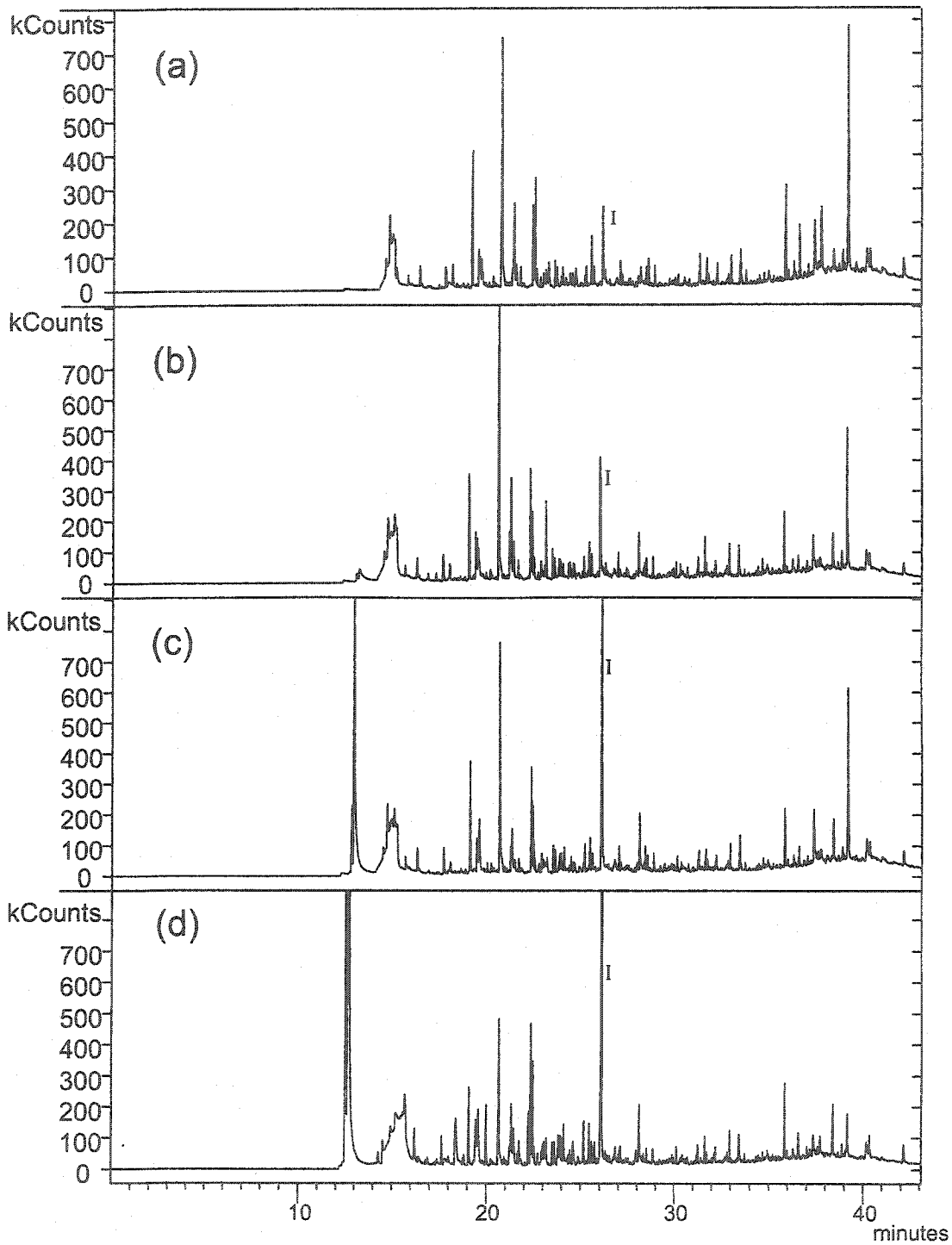


Figure 6.6 Pyrograms showing the total ion current of the lysozyme-glucose incubated samples for (a) 2 days; (b) 5 days; (c) 10 days; (d) 14 days. Peaks containing the M^+ ion at m/z 144 are designated by I.

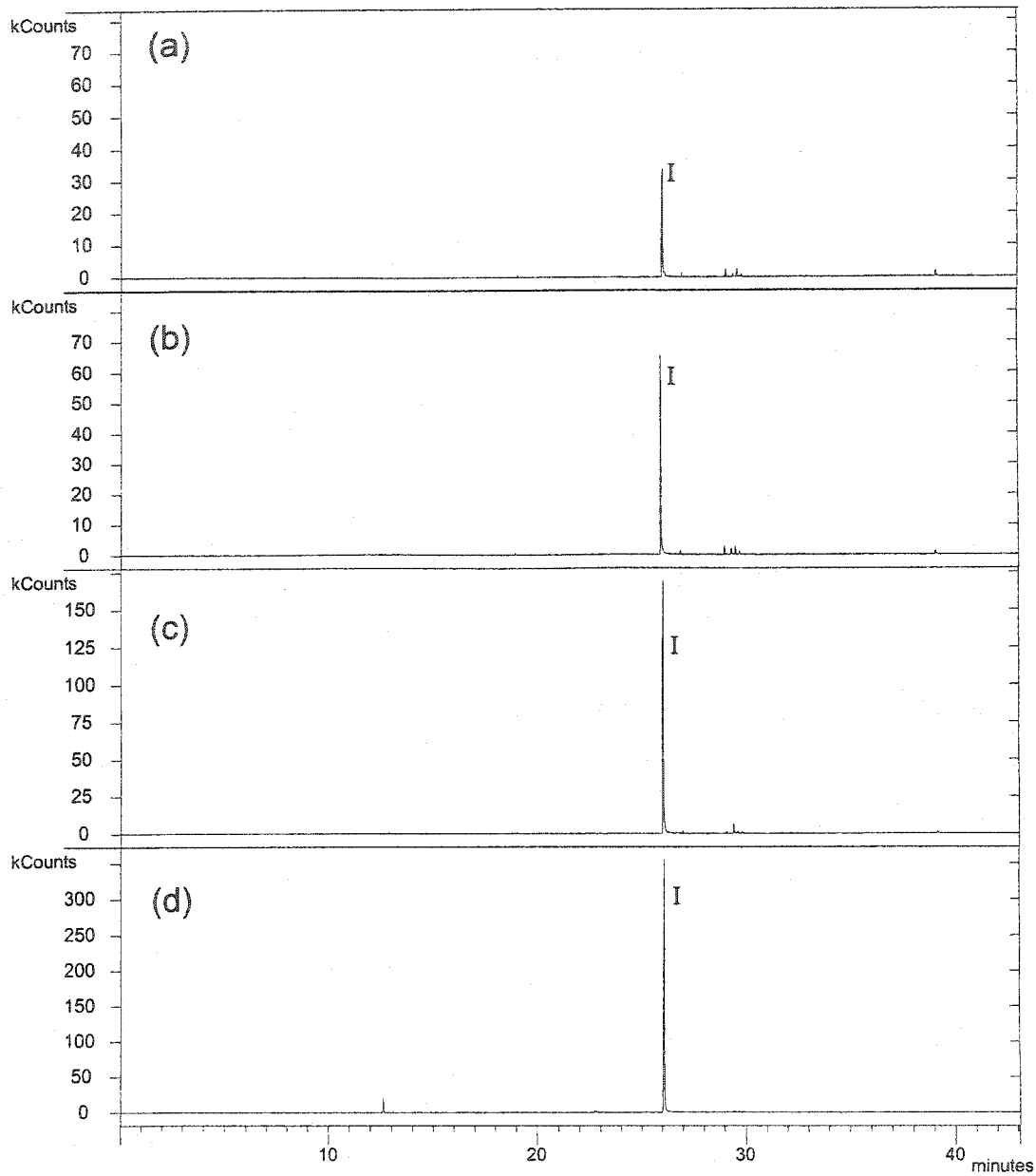


Figure 6.7 Extracted Ion chromatograms (m/z 144) of the lysozyme-glucose samples being incubated for (a) 2 days; (b) 5 days; (c) 10 days; (d) 14 days. Peaks containing the M^+ ion at m/z 144 is designated by I.

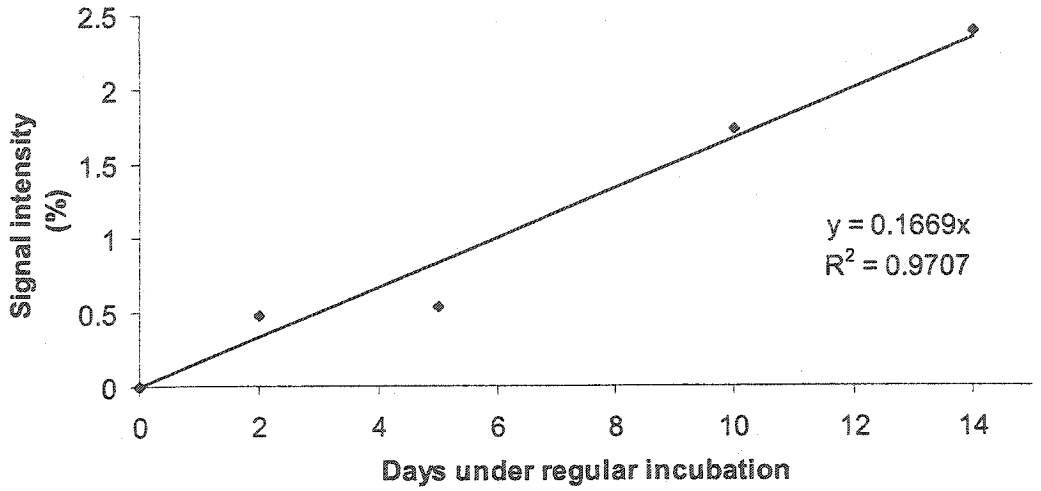


Figure 6.8 Correlation of the time of the lysozyme samples being incubated at 50°C over a period of 14 days versus the signal intensity of the diagnostic peak after deconvolution.

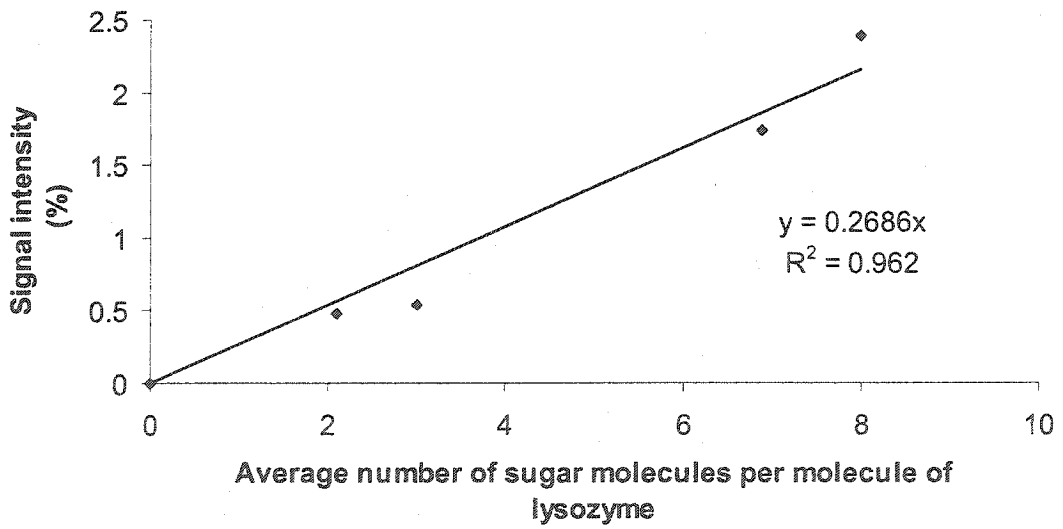


Figure 6.9 Correlation of the average number of sugar molecules attached per molecule of lysozyme versus the signal intensity of the diagnostic peak after deconvolution.

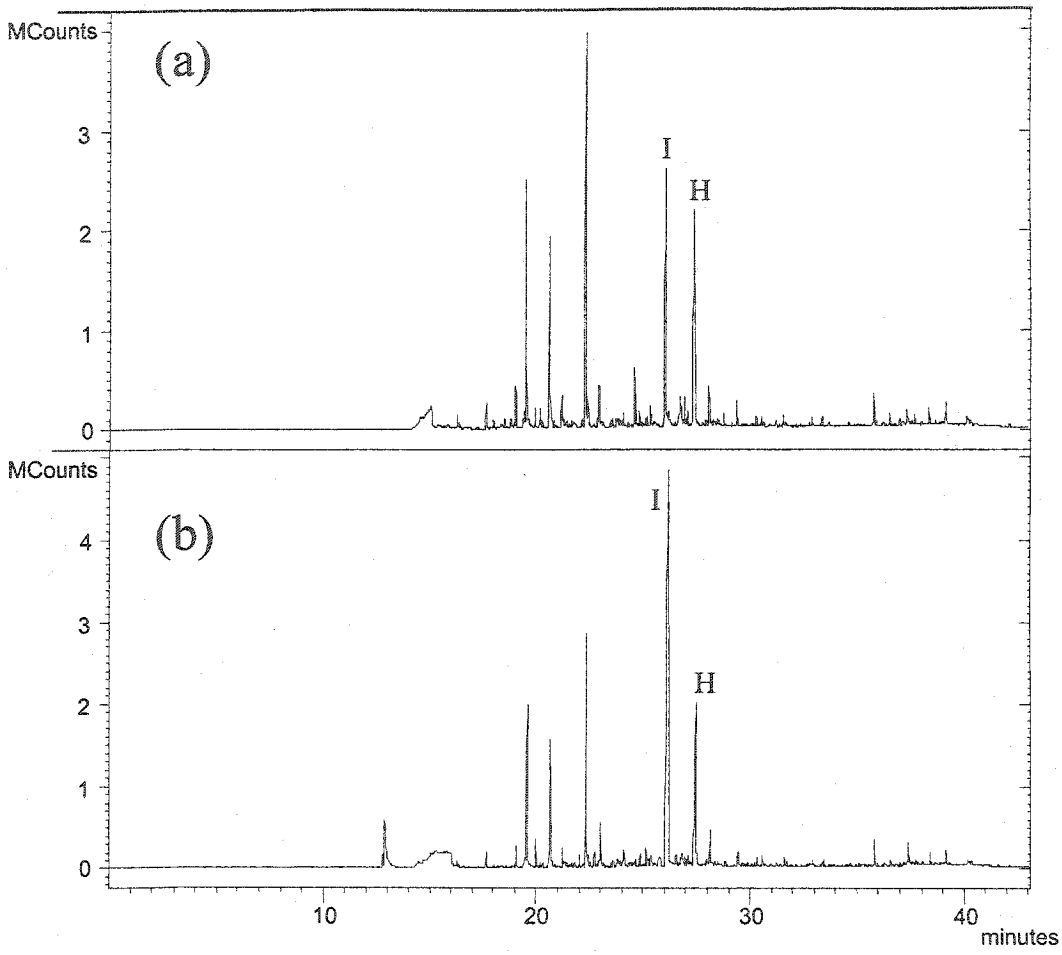


Figure 6.10 Pyrograms showing the total ion current of the lysozyme samples that had been subjected to (a) 8 hours of HHP and (b) 10 minutes of focused microwave irradiation. Peaks containing the M^+ ion at m/z 144 and the ion from HMF at m/z 126 are designated by I and H respectively.

Table 6.3 Amount of GHSA pyrolyzed and the corresponding signal intensity of the diagnostic peak after deconvolution

Amount of GHSA (mg)	Signal intensity (%)	
	First of the duplicate	Second of the duplicate
0.1918	0	0
0.3636	0	0
0.4043	0	0
0.6190	0	0
0.7955	0	0
1.2075	0	0
1.47074	0.00697[1]	0.00768[1]

[1] Amount of deconvoluted component (2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one) relative to the integrated total ion count for the entire chromatogram

Table 6.4 Comparison of treated lysozyme samples with lysozyme/glucose based on %purity and %fit, and the corresponding Similarity Index (SI).

Reference (R)	Item being compared with R	% Purity	%Fit	SI
Lysozyme + glucose (**R1**)	**R1**	**100**	**100**	**100**
	MW[2]	85.5	90.9	88.2
	HHP[3]	71.3	81.7	76.5
	Glucose	57.7	67.1	62.4
	LG-2	53.7	64.4	59.1
	Lysozyme	47.5	77.7	62.6
LG-2 (**R2**)	**R2**	**100**	**100**	**100**
	MW[2]	52.3	61.6	57.0
	HHP[3]	60.8	73.4	67.1
	Lysozyme	62.5	76.6	69.6
	LG-5	87.6	92.6	90.1
	LG-10	79.5	88.8	84.2
	LG-14	55.2	65.2	60.2

[1] similarity index = (%Purity + %Fit)/2)
[2]lysozyme sample subjected to 10 min of microwave irradiation
[3] lysozyme sample subjected to 8 hours of HHP

7 GENERAL CONCLUSION

Generation of glycated proteins with high energy exposure was investigated in this study. Lysozyme was found to have its extent of glycation accelerated with the application of HHP and focused microwave irradiation. BSA, on the other hand, resulted in lower extent of glycation under both treatments as compared to regular incubation. Glycated BSA might have undergone reversible reactions followed by the formation of crosslinks resulting in extensive denaturation to the protein with limited glycation. Application of the high energy exposures can therefore, be beneficial or detrimental to the protein depending on the nature and size of the specific proteins under investigation. Pyrolysis-GC/MS analysis on the other hand, serves as a rapid and reproducible technique to assay glycated proteins, using the diagnostic marker compounds generated through pyrolysis. Similarities between pyrograms can be assessed using the concept of similarity index, to determine the extent of structural changes in the glycated proteins.

Future work to be done include more detailed calculations of the protein content and the extent of glycation. Processing time under HHP and focused microwave irradiation on the protein-sugar mixtures can be shortened so as to better detect the optimum extent of glycation with the least damage to the proteins. Validation on the assumptions drawn on pyrolysis should be performed, so that it could be used as a fast technique in determining the extent of glycation of proteins.

APPENDICES

A1 TABLES OF RAW DATA

Raw data obtained from Lowry tests and fluorescamine assays of the different treatments are tabulated in this section.

A1.1 LOWRY TEST OF LYSOZYME SAMPLES

Table A1.1 Absorbance at 540 nm of the lysozyme incubation samples with no sodium azide

Sample	Absorbance at 540 nm		
	1st	2nd	3rd
0-1	0.681	0.689	0.690
0-2	0.739	0.752	0.755
0-3	0.785	0.795	0.796
6-1	0.258	0.261	0.264
6-2	0.260	0.263	0.267
6-3	0.248	0.254	0.255
8-1	0.298	0.304	0.306
8-2	0.282	0.289	0.290
8-3	0.280	0.287	0.288
10-1	0.282	0.288	0.290
10-2	0.277	0.284	0.287
10-3	0.269	0.273	0.277
12-1	0.297	0.302	0.305
12-2	0.309	0.314	0.321
12-3	0.291	0.295	0.304
c6-1	0.269	0.276	0.273
c6-2	0.282	0.285	0.284
c6-3	0.279	0.267	0.265
c8-1	0.224	0.239	0.237
c8-2	0.247	0.248	0.244
c8-3	0.262	0.263	0.263
c10-1	0.230	0.230	0.226
c10-2	0.231	0.231	0.231
c10-3	0.228	0.229	0.230
c12-1	0.220	0.218	0.215
c12-2	0.222	0.224	0.223
c12-3	0.225	0.222	0.223

Table A1.2 Absorbance at 540 nm of the lysozyme incubation samples with 0.01% sodium azide

Sample	Absorbance at 540 nm		
	1st	2nd	3rd
0-1	0.862	0.879	0.882
0-2	0.888	0.907	0.913
0-3	0.814	0.832	0.834
2-1	0.858	0.874	0.877
2-2	0.839	0.863	0.868
2-3	0.856	0.872	0.874
6-1	0.859	0.880	0.881
6-2	0.794	0.813	0.813
6-3	0.852	0.871	0.878
8-1	0.829	0.840	0.844
8-2	0.856	0.873	0.877
8-3	0.795	0.802	0.803
10-1	0.843	0.863	0.864
10-2	0.822	0.837	0.838
10-3	0.836	0.854	0.856
12-1	0.759	0.767	0.767
12-2	0.890	0.908	0.907
12-3	0.819	0.840	0.841
c0-1	0.913	0.919	0.923
c0-2	0.981	0.995	0.996
c0-3	0.893	0.900	0.901
c2-1	0.871	0.879	0.881
c2-2	0.773	0.775	0.775
c2-3	0.855	0.866	0.866
c6-1	0.855	0.867	0.871
c6-2	0.804	0.810	0.812
c6-3	0.930	0.937	0.939
c8-1	0.925	0.931	0.932
c8-2	0.949	0.953	0.956
c8-3	0.934	0.940	0.940
c10-1	0.902	0.913	0.912
c10-2	0.859	0.868	0.868
c10-3	0.895	0.904	0.905
c12-1	0.951	0.960	0.961
c12-2	0.847	0.851	0.851
c12-3	0.842	0.856	0.858

Table A1.3 Absorbance at 540 nm of the lysozyme samples that have been subjected to HHP at 400 MPa

Sample	Absorbance at 540 nm		
	1st	2nd	3rd
8-1	0.585	0.586	0.588
8-2	0.531	0.538	0.539
8-3	0.534	0.545	0.547
24-1	0.540	0.551	0.552
24-2	0.541	0.548	0.549
24-3	0.530	0.541	0.543
48-1	0.559	0.561	0.561
48-2	0.540	0.544	0.545
48-3	0.528	0.533	0.533
c8-1	0.592	0.602	0.603
c8-2	0.586	0.595	0.595
c8-3	0.574	0.584	0.584
c24-1	0.617	0.627	0.630
c24-2	0.586	0.597	0.602
c24-3	0.575	0.585	0.596
c48-1	0.599	0.606	0.609
c48-2	0.572	0.579	0.580
c48-3	0.572	0.582	0.586

Table A1.4 Absorbance at 540 nm of the lysozyme samples that have been subjected to microwave irradiation from 10 minutes to 60 minutes

Sample	Absorbance at 540 nm		
	1st	2nd	3rd
10-1	0.334	0.345	0.347
10-2	0.374	0.389	0.392
10-3	0.351	0.368	0.372
20-1	0.382	0.398	0.402
20-2	0.444	0.454	0.457
20-3	0.464	0.484	0.488
30-1	0.424	0.442	0.448
30-2	0.481	0.501	0.506
30-3	0.441	0.450	0.451
40-1	0.389	0.414	0.421
40-2	0.363	0.390	0.400
40-3	0.427	0.446	0.451
50-1	0.391	0.410	0.415
50-2	0.432	0.451	0.456
50-3	0.434	0.454	0.459
60-1	0.433	0.451	0.459
60-2	0.410	0.429	0.438
60-3	0.441	0.456	0.461
c10-1	0.473	0.487	0.492
c10-2	0.512	0.526	0.532
c10-3	0.515	0.540	0.548
c20-1	0.447	0.456	0.459
c20-2	0.559	0.568	0.572
c20-3	0.544	0.557	0.561
c30-1	0.434	0.459	0.469
c30-2	0.454	0.467	0.472
c30-3	0.467	0.479	0.484
c40-1	0.455	0.475	0.482
c40-2	0.286	0.295	0.295
c40-3	0.502	0.519	0.529
c50-1	0.466	0.483	0.494
c50-2	0.533	0.554	0.557
c50-3	0.458	0.467	0.473
c60-1	0.601	0.628	0.639
c60-2	0.539	0.560	0.570
c60-3	0.460	0.469	0.474

A1.2 FLUORESCAMINE ASSAY OF LYSOZYME SAMPLES

Table A1.5 Relative fluorescence of the lysozyme incubation samples with no sodium azide added

Sample	Relative fluorescence		
	@ min 1	@ min 3	@ min 5
0-1	93.3	100.3	101.9
0-2	88.3	94.2	95.1
0-3	97.4	104.2	103.1
6-1	15.0	15.7	15.7
6-2	16.7	17.3	17.8
6-3	15.3	15.7	15.7
8-1	18.9	19.9	19.8
8-2	20.0	20.6	20.4
8-3	19.0	19.3	19.4
10-1	15.9	16.5	16.3
10-2	13.6	14.0	13.7
10-3	18.2	18.9	18.7
12-1	18.7	18.6	18.7
12-2	17.8	18.4	18.4
12-3	16.5	17.1	17.0
c6-1	30.2	31.5	31.0
c6-2	29.0	30.6	30.0
c6-3	27.1	28.4	27.6
c8-1	27.1	28.0	27.8
c8-2	26.9	28.1	27.2
c8-3	25.5	26.6	26.4
c10-1	25.1	25.7	25.5
c10-2	24.9	25.7	25.5
c10-3	23.7	23.8	23.3
c12-1	20.1	21.3	20.5
c12-2	19.9	20.3	20.0
c12-3	20.1	20.5	19.9

Table A1.6 Relative fluorescence of the lysozyme incubation samples with 0.01% sodium azide

Sample	Relative fluorescence		
	@ min 1	@ min 3	@ min 5
0-1	55.5	58.7	58.5
0-2	63.0	68.0	68.0
0-3	56.9	60.7	59.5
2-1	61.7	65.0	64.9
2-2	59.5	63.1	63.2
2-3	44.7	47.0	46.1
6-1	55.6	58.7	58.1
6-2	55.4	58.4	57.6
6-3	55.8	58.3	58.3
8-1	58.8	63.1	62.1
8-2	55.1	56.9	56.8
8-3	50.2	52.5	52.3
10-1	57.5	60.2	59.6
10-2	54.9	58.6	56.7
10-3	53.4	56.2	55.7
12-1	44.1	46.7	46.4
12-2	60.9	64.8	64.9
12-3	55.3	58.6	57.0
c0-1	60.1	62.5	62.2
c0-2	63.6	67.1	68.3
c0-3	57.2	61.8	61.7
c2-1	58.5	61.8	61.6
c2-2	52.3	53.9	53.1
c2-3	61.7	64.9	62.4
c6-1	59.2	62.2	59.3
c6-2	56.2	58.4	58.9
c6-3	61.8	65.3	62.9
c8-1	61.9	65.3	64.6
c8-2	51.8	53.5	55.6
c8-3	55.5	57.5	58.2
c10-1	59.4	62.8	62.9
c10-2	56.0	59.3	58.0
c10-3	58.6	61.0	57.8
c12-1	61.5	64.4	65.1
c12-2	53.3	55.6	55.3
c12-3	56.0	58.4	58.8

Table A1.7 Relative fluorescence of the lysozyme samples being subjected to HHP

Sample	Relative fluorescence		
	@ min 1	@ min 3	@ min 5
8-1	36.0	37.0	37.6
8-2	35.7	37.8	36.4
8-3	35.1	36.7	35.7
24-1	34.7	36.6	35.6
24-2	34.8	36.7	36.5
24-3	33.6	35.5	35.8
48-1	39.2	40.6	39.2
48-2	37.5	39.6	38.8
48-3	35.2	37.2	37.0
c8-1	42.0	44.6	43.9
c8-2	41.3	44.0	44.2
c8-3	40.8	43.6	43.8
c24-1	45.2	47.6	47.1
c24-2	41.7	44.0	42.9
c24-3	40.6	42.0	41.2
c48-1	38.7	40.5	39.5
c48-2	38.1	40.9	39.0
c48-3	32.6	34.7	34.1

Table A1.8 Relative fluorescence of the lysozyme samples that have been subjected to microwave irradiation

Sample	Relative fluorescence		
	@ min 1	@ min 3	@ min 5
10-1	18.6	19.5	19.2
10-2	20.1	20.9	20.6
10-3	18.4	19.2	18.8
20-1	21.3	22.0	22.1
20-2	21.1	22.3	21.8
20-3	24.4	23.5	23.3
30-1	14.8	15.1	14.9
30-2	26.7	27.8	27.7
30-3	22.7	24.4	24.1
40-1	22.5	24.2	23.3
40-2	22.6	23.8	23.0
40-3	22.0	23.0	23.2
50-1	21.2	21.4	21.7
50-2	21.4	21.8	21.9
50-3	21.8	22.5	22.3
60-1	25.7	26.3	26.4
60-2	22.5	23.7	23.4
60-3	20.0	20.6	21.0
c10-1	24.7	26.2	25.4
c10-2	29.5	29.9	29.5
c10-3	28.7	28.8	29.5
c20-1	24.6	24.9	25.1
c20-2	31.3	32.7	31.4
c20-3	33.9	34.5	34.6
c30-1	22.3	23.2	23.3
c30-2	20.6	22.6	22.6
c30-3	23.7	24.5	24.4
c40-1	21.7	21.9	21.2
c40-2	13.2	13.7	13.6
c40-3	32.6	33.1	33.3
c50-1	27.1	28.4	27.9
c50-2	31.8	32.3	32.3
c50-3	24.7	25.7	25.5
c60-1	36.1	38.8	37.5
c60-2	31.1	32.4	32.1
c60-3	23.7	24.5	23.8

A2 TABLES OF RAW DATA OF BSA

A2.1 LOWRY TEST OF BSA SAMPLES

Table A2.1 Absorbance of the BSA incubation samples from 0 to 12 days at 540 nm

Sample	Absorbance at 540 nm		
	1st	2nd	3rd
0-1	0.449	0.461	0.462
0-2	0.435	0.451	0.453
0-3	0.441	0.453	0.44
6-1	0.397	0.407	0.410
6-2	0.438	0.438	0.448
6-3	0.421	0.429	0.432
8-1	0.422	0.430	0.430
8-2	0.397	0.404	0.410
8-3	0.436	0.444	0.448
10-1	0.378	0.389	0.389
10-2	0.423	0.431	0.432
10-3	0.426	0.431	0.430
12-1	0.425	0.429	0.428
12-2	0.406	0.410	0.414
12-3	.0.345	0.352	0.358
c6-1	0.366	0.379	0.374
c6-2	0.272	0.272	0.272
c6-3	0.283	0.285	0.287
c8-1	0.384	0.389	0.391
c8-2	0.395	0.399	0.399
c8-3	0.277	0.283	0.282
c10-1	0.443	0.441	0.441
c10-2	0.386	0.392	0.393
c10-3	0.276	0.280	0.280
c12-1	0.421	0.425	0.424
c12-2	0.385	0.389	0.390
c12-3	0.274	0.277	0.269

Table A2.2 Absorbance at 540 nm of the BSA samples being subjected to HHP

Sample	Absorbance at 540 nm		
	1st	2nd	3rd
8-1	1.049	1.115	1.120
8-2	0.256	0.277	0.286
8-3	0.565	0.613	0.635
24-1	0.558	0.599	0.619
24-2	0.548	0.587	0.604
24-3	0.298	0.325	0.330
48-1	0.389	0.419	0.432
48-2	0.546	0.592	0.604
48-3	0.481	0.511	0.527
c8-1	0.276	0.290	0.296
c8-2	0.408	0.429	0.440
c8-3	0.403	0.423	0.433
c24-1	0.259	0.271	0.279
c24-2	0.354	0.374	0.386
c24-3	0.466	0.490	0.505
c48-1	0.141	0.146	0.144
c48-2	0.229	0.234	0.243
c48-3	0.285	0.29	0.307

Table A2.3 Absorbance at 540 nm of the BSA samples that have been subjected to microwave irradiation

Sample	Absorbance at 540 nm		
	1st	2nd	3rd
10-1	0.195	0.206	0.211
10-2	0.987	1.039	1.059
10-3	1.331	1.389	1.408
20-1	0.990	1.039	1.059
20-2	0.919	0.968	0.986
20-3	1.359	1.412	1.428
30-1	0.030	0.039	0.037
30-2	1.082	1.117	1.124
30-3	0.689	0.723	0.734
40-1	0.015	0.012	0.011
40-2	0.822	0.847	0.852
40-3	0.878	0.909	0.921
50-1	0.018	0.017	0.017
50-2	0.840	0.857	0.869
50-3	0.270	0.281	0.281
60-1	0.099	0.106	0.111
60-2	0.211	0.226	0.226
60-3	0.832	0.853	0.878
c10-1	0.087	0.090	0.090
c10-2	0.115	0.121	0.120
c10-3	0.420	0.437	0.444
c20-1	0.366	0.378	0.385
c20-2	0.214	0.220	0.222
c20-3	0.352	0.365	0.370
c30-1	0.300	0.307	0.314
c30-2	0.125	0.129	0.128
c30-3	0.374	0.388	0.392
c40-1	0.112	0.115	0.116
c40-2	0.107	0.111	0.110
c40-3	0.145	0.150	0.149
c50-1	0.080	0.085	0.081
c50-2	0.066	0.071	0.070
c50-3	0.128	0.138	0.142
c60-1	0.132	0.134	0.133
c60-2	0.095	0.101	0.102
c60-3	0.091	0.094	0.096

A2.2 FLUORESCAMINE ASSAY OF BSA SAMPLES

Table A2.4 Relative fluorescence of the BSA incubation samples

Sample	Relative fluorescence		
	@ min 1	@ min 3	@ min 5
0-1	84.3	88.0	88.0
0-2	81.4	84.2	84.2
0-3	80.7	84.0	84.5
6-1	54.6	56.5	57.1
6-2	45.7	47.1	47.2
6-3	56.9	58.7	59.6
8-1	54.1	56.0	56.1
8-2	50.0	51.8	52.2
8-3	51.8	53.1	53.5
10-1	44.0	45.1	45.1
10-2	50.5	52.5	52.3
10-3	51.3	52.6	53.5
12-1	52.6	53.5	54.5
12-2	42.5	43.8	44.6
12-3	47.5	49.4	49.2
c6-1	60.2	62.3	62.3
c6-2	44.0	43.7	43.5
c6-3	49.5	50.2	50.2
c8-1	62.4	63.0	63.2
c8-2	58.6	59.5	59.4
c8-3	52.0	52.9	52.9
c10-1	71.8	72.1	72.6
c10-2	65.9	66.2	66.2
c10-3	49.6	50.3	50.4
c12-1	72.9	72.8	73.7
c12-2	51.1	51.8	52.8
c12-3	50.7	52.2	52.0

Table A2.5 Relative fluorescence of the BSA samples being subjected to HHP

Sample	Relative fluorescence		
	@ min 1	@ min 3	@ min 5
8-1	79.7	80.6	80.6
8-2	83.7	88.1	92.1
8-3	42.6	42.6	43.5
24-1	51.9	54.9	51.0
24-2	38.7	40.5	41.6
24-3	14.4	14.8	15.2
48-1	25.3	26.1	26.4
48-2	31.5	32.8	33.4
48-3	33.9	41.0	35.7
c8-1	13.2	13.8	13.7
c8-2	24.6	28.8	24.9
c8-3	28.2	30.0	30.3
c24-1	14.5	15.0	14.3
c24-2	31.4	31.9	31.6
c24-3	23.6	23.8	23.3
c48-1	14.2	14.9	15.1
c48-2	14.2	15.0	14.2
c48-3	12.1	12.2	12.3

Table A2.6 Relative fluorescence of the BSA samples that have been subjected to microwave irradiation

Sample	Relative fluorescence		
	@ min 1	@ min 3	@ min 5
10-1	13.1	12.2	9.2
10-2	119.0	123.9	123.6
10-3	141.5	147.2	148.7
20-1	112.7	94.7	93.7
20-2	103.4	100.1	98.6
20-3	88.1	91.3	91.6
30-1	2.7	3.5	3.5
30-2	109.0	113.4	113.8
30-3	50.7	45.7	45.1
40-1	1.9	2.1	2.1
40-2	63.7	66.6	66.9
40-3	72.7	74.3	74.0
50-1	2.0	2.3	2.2
50-2	83.0	86.6	87.2
50-3	21.5	22.4	22.0
60-1	2.7	3.1	2.7
60-2	10.3	11.0	11.0
60-3	58.0	58.0	59.2
c10-1	8.8	8.8	9.4
c10-2	20.1	19.8	20.0
c10-3	24.1	24.6	24.6
c20-1	21.0	21.8	21.7
c20-2	11.1	11.5	11.6
c20-3	20.3	20.2	20.8
c30-1	21.3	21.8	21.6
c30-2	24.8	25.7	26.0
c30-3	14.3	14.9	15.0
c40-1	10.9	11.2	11.5
c40-2	11.2	11.6	11.6
c40-3	10.4	11.0	11.1
c50-1	8.0	8.4	8.5
c50-2	5.4	5.4	5.4
c50-3	11.4	12.0	12.0
c60-1	42.5	45.2	46.5
c60-2	9.8	8.9	9.0
c60-3	10.2	11.1	11.5

A3 SAMPLE CALCULATION

A3.1 AN EXAMPLE FOR CALCULATING THE AMOUNT OF BSA PRESENT IN THE SAMPLES THAT GELLED

Example Weight of gel of 24-1 BSA high pressure treated sample is 0.4092 g. 6.6 mL of water and 0.5 mL of 12% SDS were added to the gel with repeated vortexing and centrifuging to have the supernatant collected for analysis. The corresponding amount of BSA in the gel will be calculated in ratio to the amount of BSA in the original solution prepared for treatment (5.043 g BSA and 2.518 g of glucose in the glucose-containing solution in 19 mL of water).

Weight of BSA = 5.043 g; weight of glucose = 2.518 g

Weight of water = 19 g (taken 1 ml of water = 1 g of water)

Total weight of the original mixture = 26.561 g

➔ Amount of BSA in the gel = (0.4092 g*5.043 g) / 26.561 g = 0.0777 g

The gel dissolved in 6.6 mL of water and 0.5 mL of 12% SDS, and 30 μL of the solution was used for test.

Therefore, the amount of BSA present in the test solution (96% purity of the BSA purchased from Sigma)

= 0.0777 g / (6.6+5.5 mL) * 1000*0.96 * 0.03 mL = <u>0.3151 mg</u>

The same calculation applied to the microwave samples, substituting the weight of the gel with the dilutions done to the original concentration prior to being irradiated.

A3.2 STEPS IN CALCULATING THE EXTENT OF GLYCATION

1. First, normalize all the protein amounts to 0.1 mg for all the samples, to get the corresponding absorbance of the samples in the Lowry test.

2. Taking the absorbance of day 0 glucose-containing samples as 100%, calculate the respective % of protein content for all the other samples and the corresponding amount of protein in mg present in the test solution.

3. Normalize all the fluorescence readings to 0.1 mg of protein with respect to the amount of protein present in the test solution.

4. Assuming that the day 0 did not undergo any denaturation upon preparation, its measured fluorescence is taken to be the expected fluorescence at day 0.

5. Calculate the expected fluorescence of the other samples with respect to the amount of protein of that test solution calculated from Lowry test and the expected fluorescence at day 0.

6. Calculate the difference in fluorescence by subtracting the measured fluorescence of the test solution from the expected fluorescence calculated.

7. Calculate the number of lysyl groups present in the day 0 test solution.

8. Then, calculate the number of lysyl groups that got glycated or denatured from the difference in fluorescence.

9. Calculate the extent of glycation by dividing the number of lysyl groups that got glycated or denatured with the total number of lysyl groups present in day 0.

A3.3 EQUATIONS FOR THE CALCULATIONS

1. Amount of soluble protein determined from Lowry test = protein content (%) * amount of soluble protein of day 0 test solution/ 100

2. Expected fluorescence= amount of protein determined from Lowry test * measure fluorescence of the day 0 test solution / amount of protein of day 0 from Lowry test

3. Difference in fluorescence = Expected fluorescence – measured fluorescence

4. # lysyl groups got glycated or denatured = difference in fluorescence * # lysyl groups of day 0 test solution / measured fluorescence of day 0 test solution

5. Extent of glycation or denaturation = # lysyl groups got glycated/denatured/ total # of lysyl groups of day 0 test solution * 100

6. Average # of reacted lysyl groups = # lysyl groups got glycated or denatured / total # of lysyl groups in day 0 test solution * the number of lysyl groups present in 1 mole of the native protein

A3.4 AN EXAMPLE IN CALCULATING THE EXTENT OF GLYCATION

Example: 6-1 of lysozyme sample (that contained glucose).

The normalized absorbance is 0.350. With reference to the absorbance of the day 0 sample (0.895, which is assumed to have 100% protein content), protein content of the day 6 sample is 0.350*100/0.895 = 39.16% and the corresponding amount of protein present is 0.03916 mg (with reference to 0.1 mg in the day 0 sample).

Normalized fluorescence of day 0 sample is found to be 117.5, and that of day 6 sample is 21.8. Therefore, the expected fluorescence that should have with 0.03916 mg of protein should be 46.0 (0.03916 mg * 117.5 / 0.1 mg). The difference in fluorescence between the measured fluorescence of the day 6 sample and the expected fluorescence is 24.21. In other words, the difference in fluorescence refers to the lysyl groups that got glycated by the glucose, and this could be used to extent of glycation by dividing it with the original number of lysyl groups present in the day 0 sample.

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