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Chemical Changes seen in Protein during Pelleting – a literature review

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Feed Manufacturing Technology

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Abstract

Pelleting induced changes in protein denaturation with exposure to heat is to milder level. Denaturation of protein showed better digestibility of protein molecule. Change in the secondary structure i.e. alpha helix, beta sheet, beta turn and random coil is seen during pelleting and pressure induced treatment. Secondary structure directly affects the solubility and digestibility of the fractionated protein. Increase in conditioning time highly affect the fragmentation of protein molecule. As the heat is applied the molecular dissociation of the protein molecule lead to fractionated protein of smaller molecular weight. The amount of smaller molecular weight is higher compared to large molecular fraction. Antinutrients level is diminished to certain extent on exposure of heat during pelleting. Some of the amino acids like lysine and cysteine is prone to undergo maillard reaction and form Amadori compound. Degradation of lysine is higher during pelleting. Increase in degradation of lysine rises with shifting of the medium towards alkaline scale.

Keywords: Pelleting, Secondary structure, Denaturation, Solubility, Conditioning time, Small molecular weight and Lysine.

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Author

Shiv Nath Shah

Introduction:

In ancient time, pelleting was done under uncertain measurement resulting in unscientific quality of the feed. Pelleting is considered among one of the oldest means of feed processing technique (Menno Thomas, 1998). During early time considerable study on animal nutrition was lacking. Most of the animals were kept free to graze, so that they can search food for themselves or the products from households and/or by-products from food industry were fed. Also in earlier time of Napoleon, agglomerated feed made with expeller type machine was in use for feeding the horses (Menno Thomas, 1998). Pre-treatment or conditioning was introduced before pelleting of the feed mash to improve the physical quality (hardness and durability) of pelleted feed (Menno Thomas, 1998). During this time water was only the solvent system used for conditioning. Due to advancement in instrumentation the varieties of solvent system were implemented in conditioning with conjunction to shear forces (Menno Thomas, 1998). The use of steam, molasses and other solvents is increasing day by day.

Modern livestock feed manufacturing requires diversified raw materials to makeup compound feed. A profound knowledge is required on these raw materials in selection of different property based nutritional value of the feed (M. Thomas & A. F. B. van der Poel, 1996). The mixture of raw materials with distinct characteristics are forcefully pushed in the circular metallic die through mechanical means. Heat, moisture and pressure is also used to shape it in to harder and larger particles. These pelleted, compact larger ones make ease in handling, palatability and enhanced feeding aftermath. Feeding of pellets is found to be positive in all livestock grower. There are number of reason behind this; a) heat produced during conditioning and pelleting turns them into more digestible stuff, b) align the feed in compact form, and c) pelleting reduces the wastage in the time of feeding. Selection of proper diet is benefited over picking different ingredients for animals. Feed conversion in supplementation of probiotics is seen to be enriched by 2.3% in broiler industry (Amerah et al., 2013).

Protein are crucial element of our eco system. They are derived from the “Greek word proteois”, defining the word for the first kind. It is distributed throughout the cell from plasma membrane to the inner nuclear structure. Proteins are entirely a complex molecule, or we can say polymer with three-dimensional structure. These structural complexities give them the potential to show different functional properties. Proteins are synthesized form the primary 20 amino acids. The sequence in which these amino acids are arranged, determines the structure and functionality of the protein. Amino acids are linked to each other through covalent bond known as peptide bond. Disulphide bonds present intramolecularly and/or intermolecularly giving the proteins

different folds. In the native stage, proteins are surrounded by different attractive and repulsive forces. These forces are responsible for the stability of protein in environment of presence i.e. in the native state. Exposure of the temperature and mechanical shear during pelleting leads to change in the molecular structure.

Aim of the Thesis:
The aim of this thesis is to increase the understanding of chemical changes observed in protein during pelleting of composite feed ingredients. The degree of change in the chemical structure of macromolecules especially protein during formulation under steam and temperature.

Pelleting Process:

Manufacturing of the feed for animal undergoes through distinct phases during processing. Commonly used sequence in manufacturing of feed by feed industries are: receiving of raw materials, grinding or particle size reduction, proportioning or batching, mixing, heating or thermal treatment, packaging, warehousing and loading (Abdollahi, Ravindran, & Svihus, 2013).

Grinding:
Grinding is the initial step after receiving and cleaning of the raw material. Although, grinding reflect noticeable cost in energy means, it is necessary to overcome or change the physical state of the ingredients (Svihus et al., 2004). It is done for cereal grains to attain the uniformity in particle size and to control the rheological properties in the mixture of ingredients. Grinding helps to increase the surface area altering in the physical characteristics of the feed ingredients along with availability of nutrient for digestion. Apart from the physiological function of grinding particle size reduction play significant role in technological properties like binding, texture development, hardness during conditioning, pelleting, expansion and extrusion process. As the surface area of the particle increase during grinding the ability to absorb water during pelleting increases (Behnke, 2001). This will aid in gelatinization of starch along with plasticizing of the protein molecule. Finally reduces the gap between the particles giving better binding effect and enhance hardness of the pellet and reduction in the fines (M. Thomas & A. Van der Poel, 1996). Most commonly used mill for size reduction are hammer mill and roller mill.

Hammer Mill:
Hammer mill is very common in the feed industry. Several narrow hammers or hitters made up of high tensile steel is either fixed or in hanging position to a rotor. This rotor is assembled

within steel frame. Hammer mill can be turned on both side i.e. one side is impact one while the other side is striking force. The intensity for milling is dependent upon nature of the material and amount being loaded in the mill. It also contains a steel screen which determines the size of particle. One or more number of magnet is placed to inhibit the metal particle entrance in the feeding chamber. Presence of metals in the feed grains can physical damage to the hammer system.

Roller Mill:

Roller mills are not in common use as hammer mills. Roller mills fives uniform particle size with lesser number of fine particles compared to hammer mill. During operation roller mill is sound friendly compared to hammer mill. Grinding results for fibrous cereals and natural mixed grains is not satisfactory regarding roller mills. The space between two rolls determines the particle size reduction.

Conditioning:

Conditioning is a thermomechanical process in which heat, water, pressurized steam and time is used to convert the mixed feed mash in to a physical form which is desirable for compaction during pelleting. It is considered of being crucial part performed prior to pelleting during pellet processing. The processing time depends on the component used in the feed mix. It usually processes for 20 to 30 seconds. In this process steam (moist heat) and molasses is added to the concentrate feed mash. The percentage of moisture addition is of vital importance as it aids in agglomeration of the feed mixture, finally giving the durability of the pellet. Conditioning acts as a soothing affect for the feed mix and reduces the friction between the die while coming out through it. High Production rate at low cost can be achieved with conditioning along with reduction in energy consumption.

The amount of steam added during conditioning varies upon different formulation, temperature, etc. Formulations can be divided into diverse types or categories taking protein content as consideration. High protein feeds, such as supplements and concentrates require more steam to gain the plasticity of the protein. This will help to reduce the friction being developed during pelleting. Heat is also of high concern regard to moisture as it helps in pasting of the feed mash. Grain feed contains high amount of starch and is applicable for poultry, turkey, etc. both higher level of moisture and temperature is required to gain proper starch gelatinisation. This will help to get the durable pellets as it aids in better binding of the feed mash.

Pelleting:

Netherlands have production of pelleted feed with a projection value of 88.4% in a total of 16.1 million tonnes (compound feed) in the year 1993 (M Thomas, Van Vliet, & Van der Poel, 1998). Pelleting is considered as one of the most common processing unit in context of poultry feed. The pelleting process can be defined as agglomeration of different component used in feed mix into larger one making a pellet with the aid of mechanical process. Heat, pressure and moisture is added as per desired texture and shape of pellet. The conditioned feed mash coming from the conditioner is pushed into the chamber through metal die. The die used for pelleting of feed mash are generally ring shaped which can rotate on a fixed roller. When the subjected feed mash passes through the chamber, layers formation take place on the die and due to rotation of ring inside the pelleting chamber, compression of material occurs resulting in formation of compact compound feed. Starch gelatinization takes place during pelleting process, but the degree of starch gelatinization is limited to a smaller limit. Partial level of protein denaturation is also seen during this process which help in gaining better hardness and durability of the pellet (M Thomas et al., 1998). The enzyme inhibitors which are present on the native form of proteins get destroyed or inactivated during pelleting. This enhances the availability of the protein for nutrition during animal feeding. The nutritive value which are encapsulated in the endosperm of the material comes out of the cell making ease in uptake for the enzymes.

Cooling:

Pellets comes out of the die of pellet mill with a temperature from 60 to 95ºC and 120–175 g/kg of moisture content (M Thomas et al., 1998). Cooling is done to lower down the level of moisture content and temperature (heat content). Also cooling helps in recrystallizing of soluble material which makes a bond within some feed composite particles. Also, association of protein molecules take place during cooling (M Thomas et al., 1998). Air drying is used to remove the surface heat and moisture from the hot pellets. The left-over fines in the cooler or dryer is collected and placed into the chamber for reprocessing. The pellets after cooling and drying, passes through the crumbler. The selection of pellets passing a crumbler is dependent upon the type of product being made.

Protein:

Protein in a native stage is a complex molecule. Knowing about the structure of the protein is crucial in the sense of getting closer in understanding the digestion and nutrition value. Availability of the protein for use in animal feeding is another important aspect of learning about protein. Protein given to animals in a raw form may hinder the availability of desirable

and well balance amount of amino acid for uptake in the intestine. This hindrance directly effects on the growth and development of animal. To overcome these disadvantages in feeding of animal several processing techniques with use of variable temperature, pressure, moisture and mechanical forces are used. These treatments of the raw protein with the use of different advanced techniques enhances the nutrient bioavailability for the animals.

Studies in this regard of protein structural changes during pelleting i.e. physical and chemical is very less. Proteins have their different helical profile ration and the higher amount of beta sheet indicates relation to nutritional value of the formulated compound feed. These ratio of the alpha helix and beta sheet affect their availability for the enzyme (protease) activity in the gut of animals. High amount of β-sheet in the feed will decrease the protein solubility and crude protein digestibility. This is due to higher number of hydrogen bond content in the β-sheets. Proteins from same source with different secondary structure directly affect the availability for the absorption due to change in alpha and beta chain ratio.

There are very less analytical methods and instruments developed to understand change in internal molecular structure during pelleting. Conventional techniques are not well suited in studying the structure of protein as in this technique hard chemicals are being used. These hard chemicals do alter the original or native stage of protein during analysing. Nowadays newer advanced form of analytical techniques is applied in studying the structural changes in a bulk feed. The use of FTIR (Fourier transform infrared reflectance), FTATR (Fourier transform attenuated total reflectance), NMR (Nuclear Magnetic Resonance), gel electrophoresis, etc in feed analysis is increasing.

Structure of Proteins:

Proteins are complex molecule with high molecular weight. Their basic structure is composed of 20 different amino acids. Among these 20 amino acids 9 are the essential amino acids which cannot be synthesized by our body (Gerrard et al., 2012).

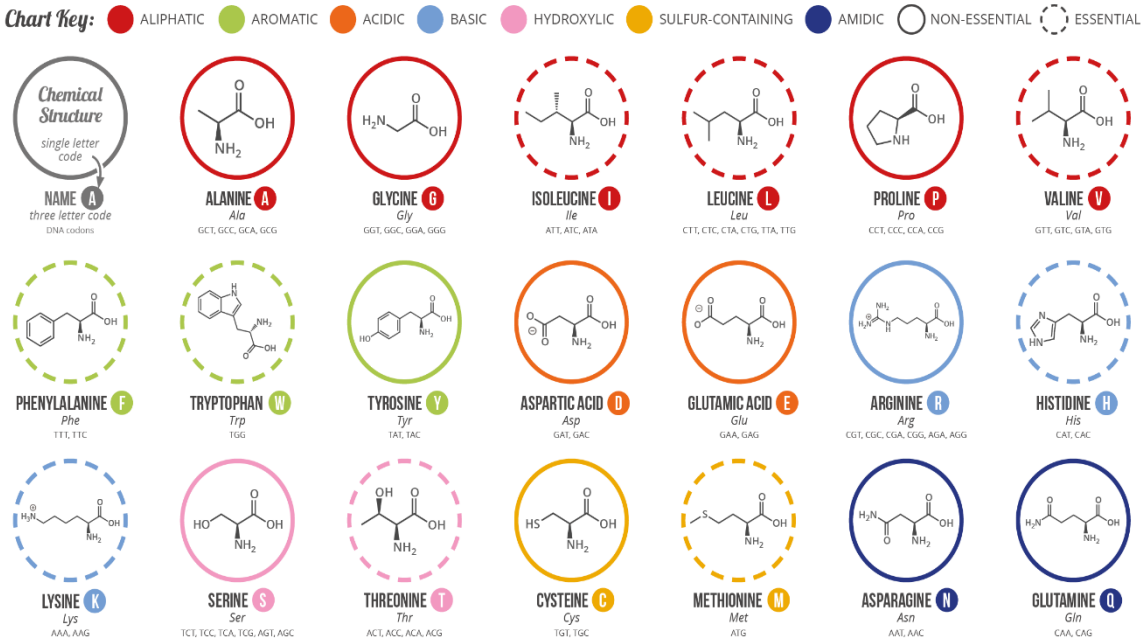


Figure 1 This figure depicts the structure of 20 available amino acids with their chemical properties (Compound interest, 2017).

These amino acids vary with each other in respect of the three-dimensional structural distribution of diverse groups. These structural configurations of amino acids in feedstuffs determines the nutritional value of the protein. Amino acids contain chemically active groups which are prone to reaction when encounters distinct groups from other macromolecules. Reactivity of such active groups depends upon different environmental factors such as temperature, pressure, PH, etc.

Primary Structure:

Primary structure is the linear linking of the amino acids through covalent bonds (peptide bonds). The carboxyl group and the amino group of the two different amino acids get bonded through loss of one molecule of water. The end having amino group is called N-terminal and the end with carboxyl group (COOH) is called C-terminal.

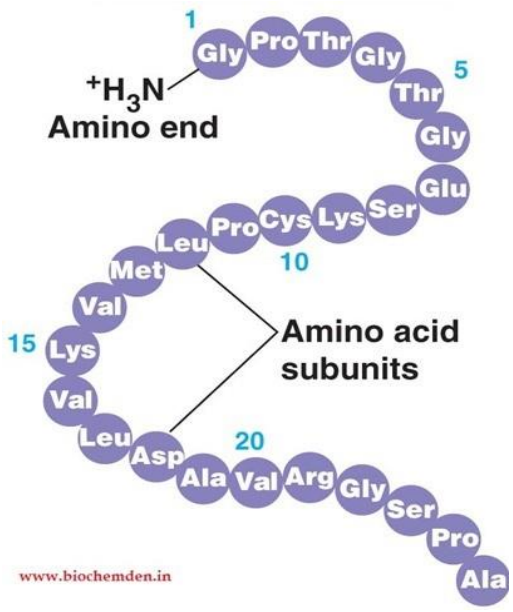


Figure 2 Basic Primary Structure of Protein (Biochem Den, 2017).

Secondary Structure:

Primary structure of proteins cannot stand stability in the native state. So, this molecule goes through folding to form a helical structure or is shaped in to a sheet. This folding is balanced with the formation of hydrogen bonds. Secondary structures always exist in alpha helical structures and beta sheets.

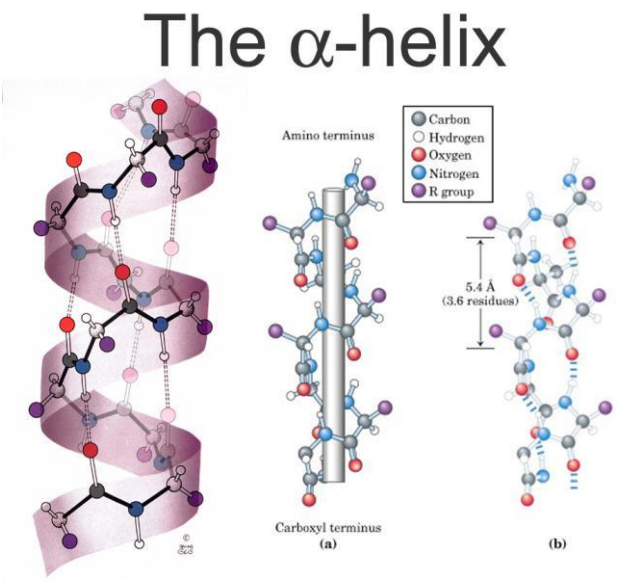


Figure 3 Helical structure of Protein (Kullabs, n.d.).

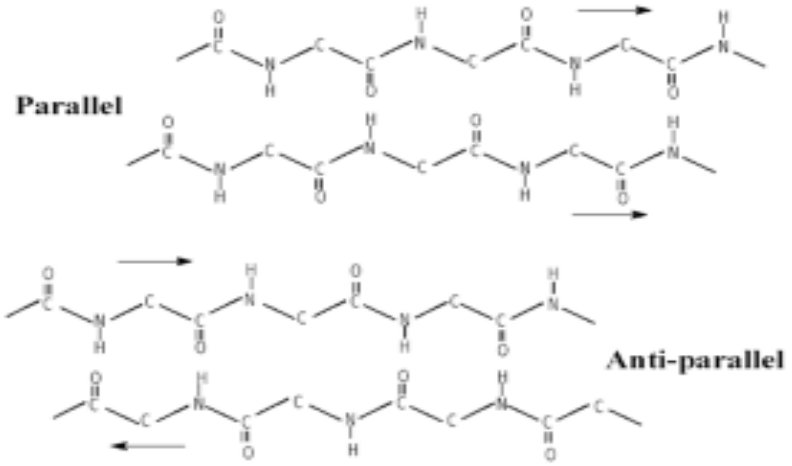


Figure 4 Protein secondary structure showing beta sheet formation (Biochemphilic, n.d.).

Tertiary Structure:

Tertiary structure is the three-dimensional spatial arrangement of the protein. These structures are maintained due to different bonds formation (disulphide, hydrogen and ionic bonds) and presence of different hydrophobic and hydrophilic interaction within the molecule. Tertiary structure is highly prone to deformation in the presence of heat during pelleting. This change in the secondary, tertiary and quaternary structures without altering the peptide bond is called denaturation. Once proteins are exposed to heat, the secondary, tertiary and quaternary structures start to disassociate to certain limit and on further heating, newer hydrogen bonds formation takes place resulting in the change in native structure of proteins. These changes are reversible to certain extent, after greater exposure to the heat the process is irreversible. The cross linking of the protein molecules with other molecules and the protein molecules itself determines the accessibility of the molecule for digestion and proper utilization as a nutrition.

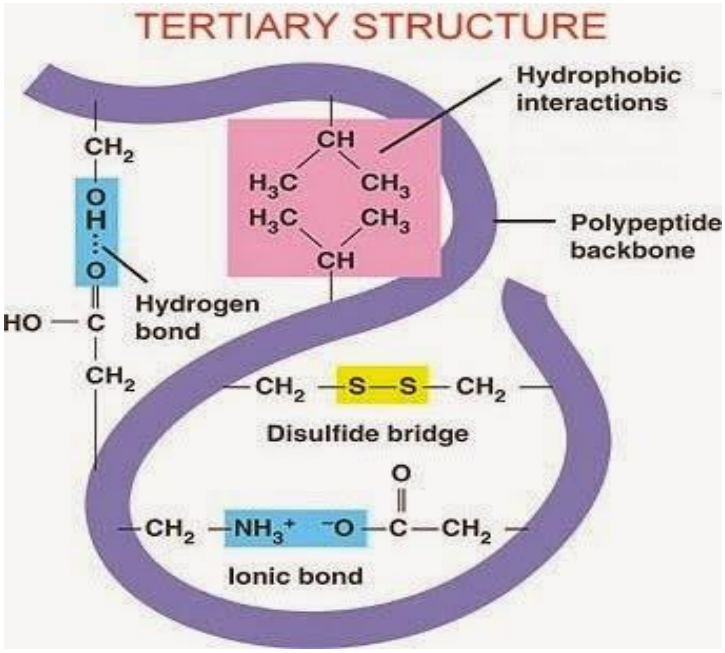


Figure 5 Tertiary structure of protein showing different bonding interactions (A Level Notes, 2016).

Quaternary Structure:

This is the complex structure of the protein molecule. Two or more polypeptides molecules binds with each other making a super coiled complex helical molecule. It is sometimes linked with inorganic element like haem (Iron containing molecule) in haemoglobin. Collagen fibres are also a good example of quaternary protein molecule as in this no of hydrogen bond formation takes place.

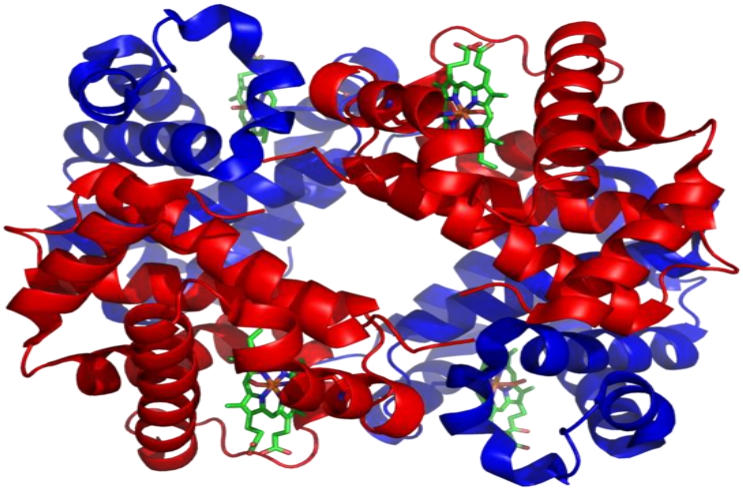


Figure 6 Quaternary Structure of Protein Showing coil formation (Biochemistry question, n.d.).

Protein Hydration:

Water acting as a solvent, dispersion medium and plasticizer in feed and food processing is a crucial factor for protein solubilization (Matveev, Grinberg, & Tolstoguzov, 2000). It changes the physiological and chemical structure of protein during feed formulation. The rheology and texture of feed depends upon the interaction with the formulating constituents particularly with proteins, polysaccharides, and water. These polymer system (protein and polysaccharides) when encounters fats and water tend to change their conformational stability (Matveev et al., 2000). Protein association and protein mesophases formation takes place in food system (Matveev et al., 2000). A globular protein molecule can be viewed as hydrophilic glassy state membrane encapsulating a hydrophobic core (Matveev et al., 2000).

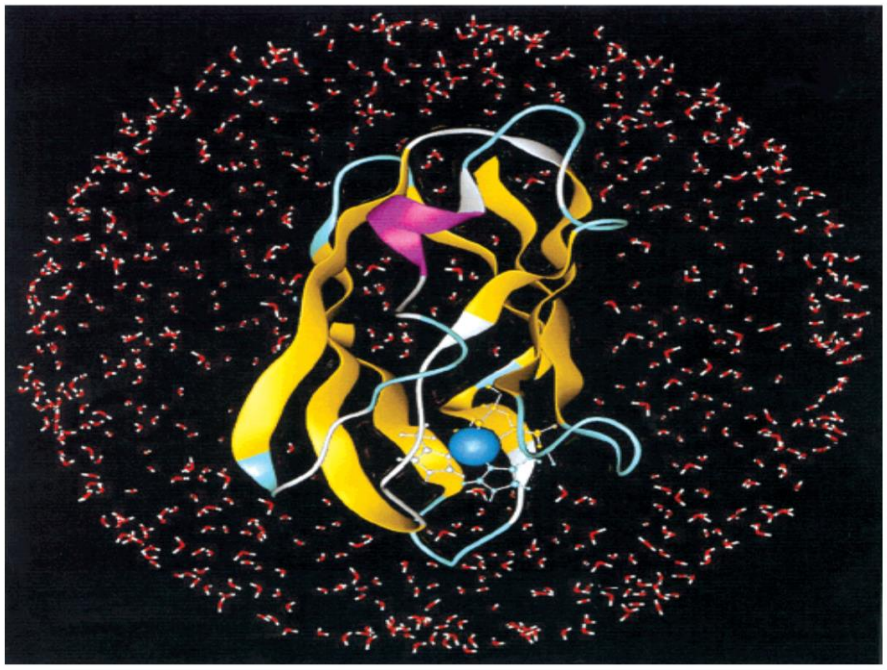


Figure 7X-ray structure of protein molecule surrounded by 3514 water molecules (Bizzarri & Cannistraro, 2002).

Functionality of Protein molecule depends on the interaction of protein with water. Water molecule interact with different charged ionic end, peptide end, amide end of Asparagine and Glutamine, hydroxyl group of Serine, Threonine and Tyrosine residues and nonpolar end of protein complexes (Damodaran, Parkin, & Fennema, 2008). Polar side of the amino acid binds with water forming hydrogen bond. N and C terminal of the amino acid is charged, and they usually make electrostatic interaction with water molecule. Hydrophobic interaction is seen in the non-polar end of the amino acid group. Protein hydration capacity is dependent upon number of charged end present in protein complex. Some of the protein like casein have colossal

amount of vacant space in their structure which absorb water in to it through capillary action (Damodaran et al., 2008).

Water can act on protein on three diverse ways: a) strongly binding internal water, b) water interacting protein interface (surface water), and c) bulk water (Bizzarri & Cannistraro, 2002). Strongly bound water shows activity in hydrogen binding with protein and solvent. Interfacial water can interact both with the protein molecule and other surrounding different molecule. Bulk water does not take part in the direct interaction with protein molecule, but it is in contact with surface water. Bulk water goes on exchange with surface water from time to time (Bizzarri & Cannistraro, 2002). Mechanical shear, heat, water used during processing of feed enhances the binding properties of the protein.

Protein Denaturation:

The structure of protein molecule in their native state is dependent upon different forces acting on it. They are: attractive forces, repulsive forces, intramolecular forces and interaction of inclosing solvent water with protein molecule. Proteins in their native stage is most stable in context of thermodynamics as there is almost absence of practicable free energy. Slight change in the structure without altering the molecular geometry of protein molecule is called conformational adaptability. While if there is a considerable change in secondary, tertiary and quaternary structure outwardly not breaking the peptide bond can be called denaturation of protein. Denatured protein losses all their physiological functioning which they can show in their native state. Many of the proteins go through structural unfolding pursued by aggregation on exposure to moist heat or mechanical shear. This structural unfolding of protein during heat treatment is reversible if the thermal and mechanical treatment is paused before aggregation of molecule starts. If the treatment is resumed, noncovalent bonds and other interactions supporting the three-dimensional structure will be crumbled leading towards irreversible protein denaturation.

Oftentimes, protein denaturation has unfavourable significance as it reveals depletion of important protein properties i.e. biological activities. Looking on to feed technology application, denaturation of protein is eminently adorable. Thermal denaturation of proteins enhances the digestibility by inactivating the enzyme inhibitors, creating a new site for enzyme action (Abdollahi et al., 2013).

Denaturation of protein induced in presence of heat during pelleting leads towards physical and chemical changes in the protein molecule. The protein native structure changes with change in

solubility and hydrophobicity. Because of ongoing hydrophobic interactions and intermolecular disulphide bond breaking and newer bond formation (rearrangement), the protein molecules go through aggregation resulting in poor protein solubility (Čolović et al., 2013).

In context of food technology application, partly denatured protein on air in water and oil in water interfaces enhances the foaming and emulsifying properties (Damodaran et al., 2008). While on extreme thermal treatment of soy protein lowers down their foaming and emulsifying properties (Damodaran et al., 2008).

Proteins have various application in food system based on their different functional Properties. In application to feed industry, proteins showing potential adhesive nature are of immense importance. Consequently, proteins considered to be good binders during feed formulation. At temperature of approx. 200K, the molecular structure of protein attains a freezing condition which is like metastable state (Bizzarri & Cannistraro, 2002). This stage is kinetically stable (arrested) and is called glass like transition.

Effect of Heat on Protein Molecule:

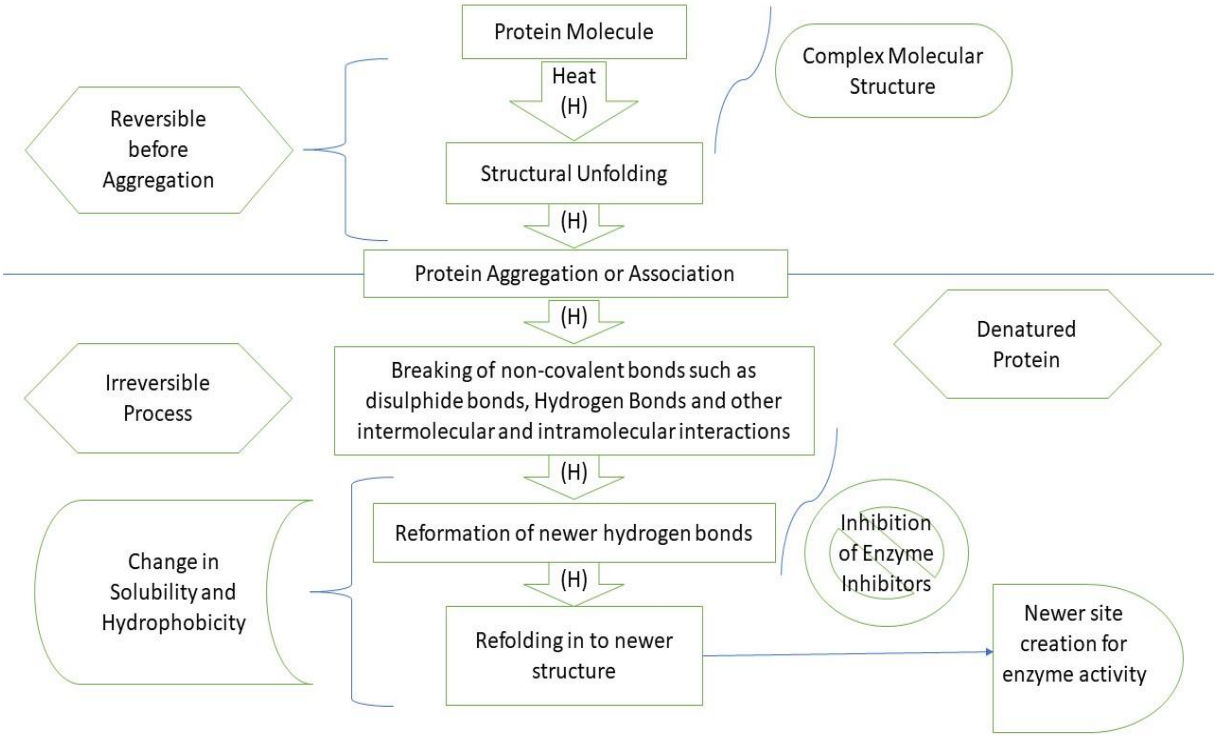


Figure 8 Chemical Changes and interactions in Protein with nutritional value in Food or Feed.

In the figure 8 placed above, change in the protein molecule on exposure to heat (Thermal processing) is shown in general. Protein in their native state is in complex molecular structure

bounded with different covalent (peptide bond) and non-covalent bonds (disulphide bond, hydrogen bond), intermolecular and intramolecular interactions. On heating, structural unfolding of the protein molecule takes place. This leads to protein partial aggregation and denaturation of protein molecule. This process is reversible if heat exposure is removed. On further heating, breaking of non-covalent bonds takes place. Once the non-covalent bonds stars breaking it enters the irreversible part of protein change. The protein molecules tend to denature making free space for reformation of newer non-covalent bonds. The opening of the tertiary and quaternary structure of protein gives free active site formation for interaction with newer functional group from other macromolecules or the neighbouring protein molecules. These interactions lead to the change in hydrophobicity and solubility of the protein molecule. Gaining of more heat form the processing instruments further leads to refolding of the molecule in to newer conformational state. Some of the known antinutrients present in the cereal grains and legumes start to get degraded on exposure to heat. This creates newer site for enzyme activity enhancing the protein digestibility. These are the beneficial point of heat treatment seen in the protein molecule. Some of the crosslinking oxidation reaction occurs during this process. These reactions may lead to racemisation (conversion of the L-levo to D-dextro amino acids). As we know that Levo form of the amino acid is only been utilized by the animals as a nutrient source. This converted Dextro form of amino acid have no physiological role in growth of animal or protein synthesis. On further treatment with heat leads to non-enzymatic reaction where free amino group of amino acids get reacted with carbonyl group of sugar molecule forming Amadori compounds. This reaction is called Maillard reaction. More detail on this reaction is mentioned in other page. Continuing the treatment with heat leads to carbonization (conversion of protein molecule in to carbon).

Spectral band seen through FTIR for different proteins:

The intensity of band obtained during IR spectroscopy of the protein can be used to analyse the content and quality of protein. The variation in the intensity of the band and the change in nature of the band distribution reflects the quality of protein (change in structural conformation of the protein), their utilization and digestion property in different species mainly in dairy cows (Bai, Qin, Sun, & Long, 2016). The peptide bond which is the functional unit of protein molecule when subjected to infrared ray gives different bands in the spectrum. Amide I and Amide II are the two potentially important bands seen in the spectrum obtained through FTIR technique of analysis.

Amide I band: The amide I band is formed due to stretching vibration of C=O group and is seen near 1650 cm^{-1} (Barth, 2007). This is directly dependent upon the backbone of the protein molecule. The presence of different functional group does not affect the intensity of amid I band. Although the secondary structure i.e. the length of the side chains effects the amide vibration. Amide I band is crucial in determining the secondary structure of the protein molecule (Barth, 2007). Secondary structure is of prime concern while studying the nutritional benefits related to protein changes or denaturation.

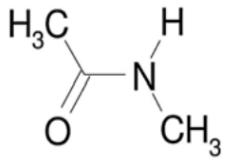


Figure 9 N-methyl acetamide structure with trans peptide group (Barth, 2007).

Amide II band: The amide II band is a result of combination of stretching and bending vibration. Here N-H bending (60%) and C-N stretching (40%) is observed which gives the formation of amide II band at 1550 cm^{-1} (Barth, 2007).

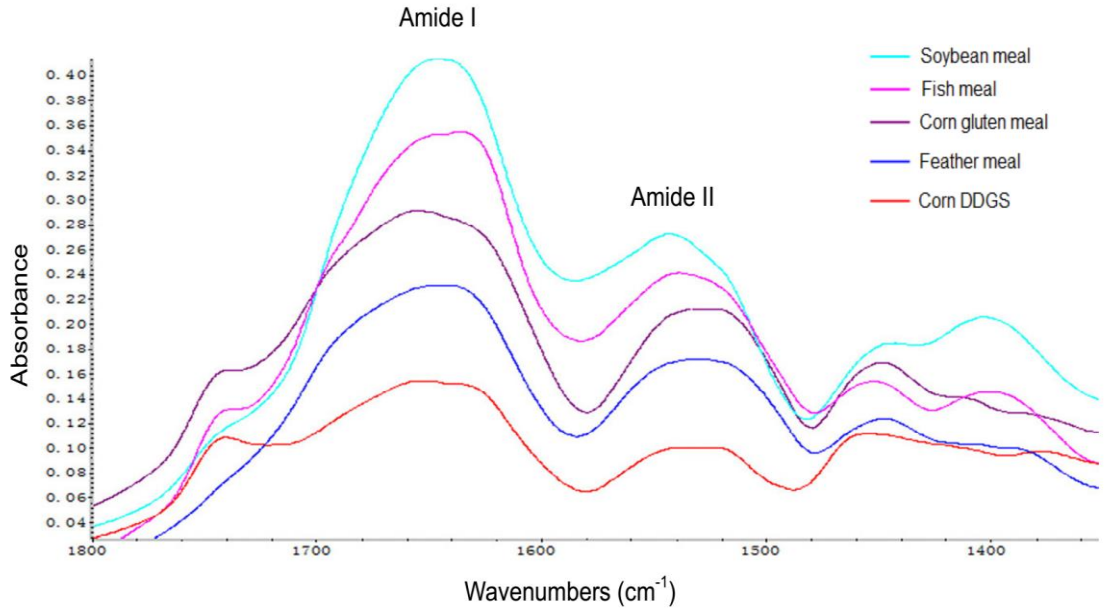


Figure 10 IR spectrum of the five different proteins showing two distinct spectral band i.e. a) amide I (1,700 to 1,600 cm–1) and b) amide II (1,600 to 1,500 cm–1) (Bai et al., 2016).

Effect of Heat and Pressure on Ovalbumin seen in Spectral band through FTIR:

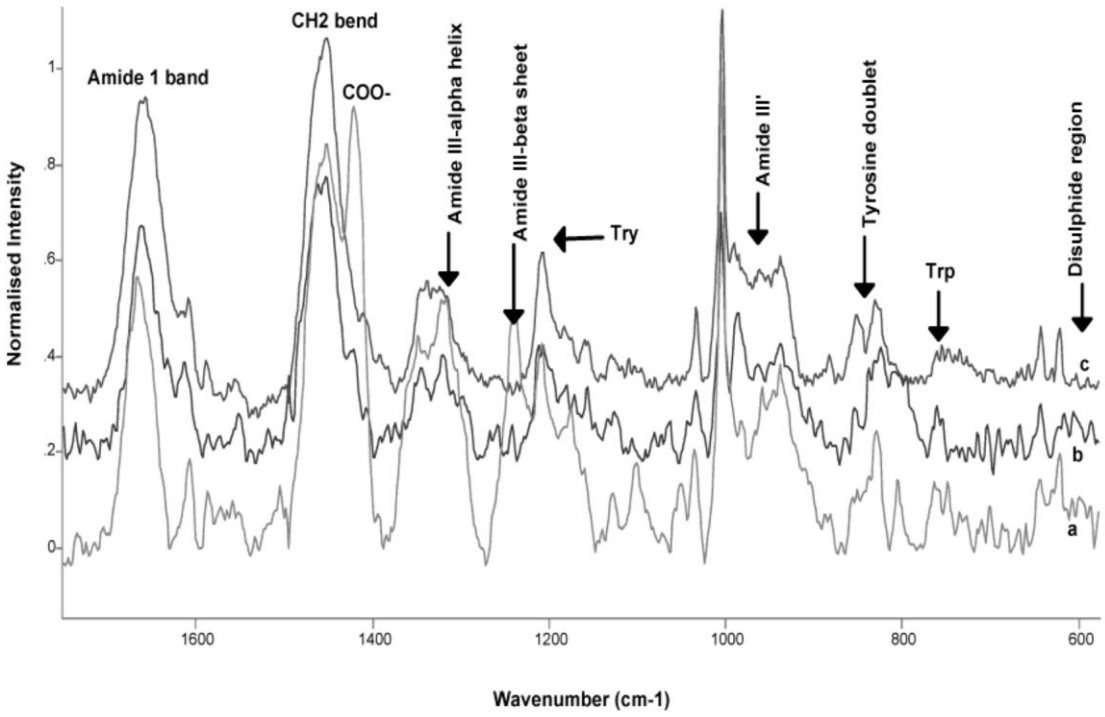


Figure 11 Spectrum of Ovalbumin in three different sample form a) native, b) heated and c) high pressure treatment (600 MPa for 20 minute)(Ngarize, Herman, Adams, & Howell, 2004).

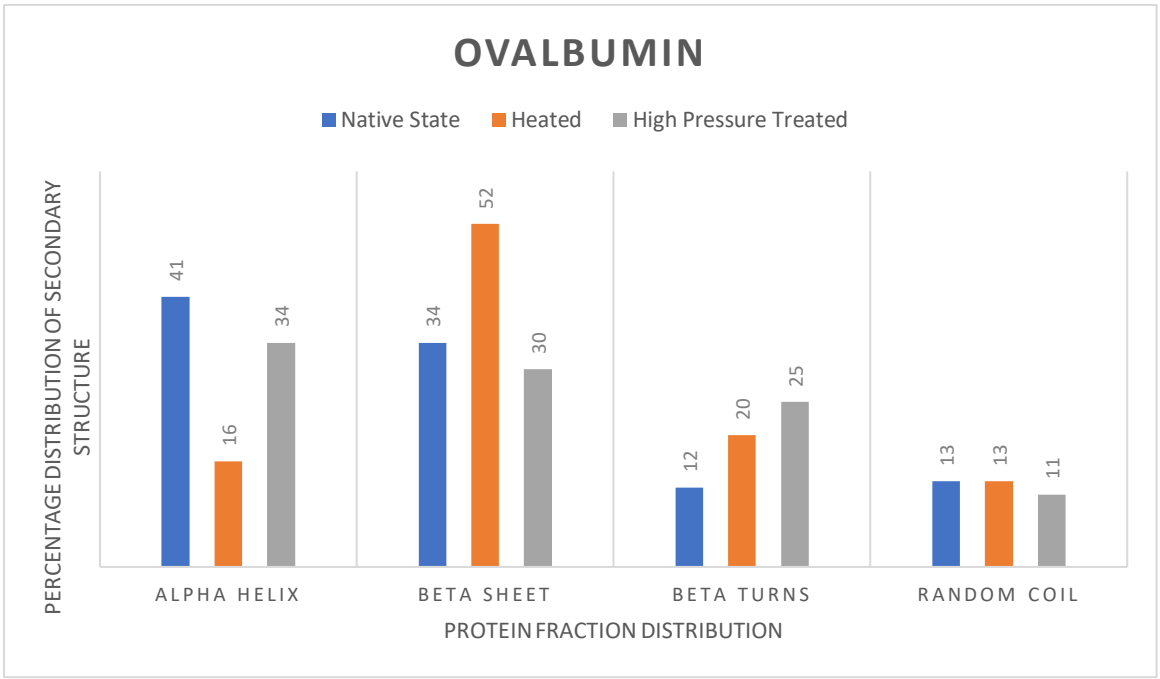


Figure 12 Fractional distribution of secondary structure of Ovalbumin protein in native, heated and high pressure treated condition (Ngarize et al., 2004).

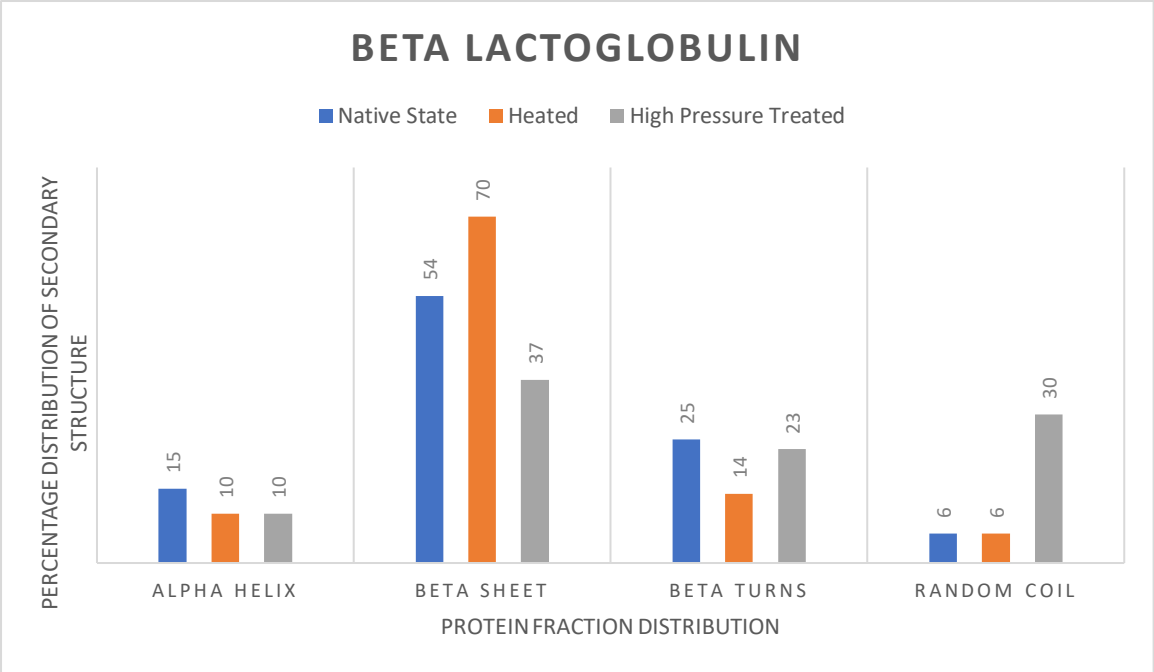


Figure 13 Fractional distribution of secondary structure of beta lactoglobulin protein in native, heated and high pressure treated condition (Ngarize et al., 2004).

Based on the study, heated samples of both protein (ovalbumin and beta-lactoglobulin) showed significant changes. There was a decline in the intensity of band 1648-1658 cm-1 (Ngarize et al., 2004). This band projects towards alpha helical structure as shown in the table. Also, band formation is seen at 1618-1620 cm-1 in both heated protein (ovalbumin and *beta*-lactoglobulin). This band reflects the breaking of hydrogen bonds and reformation of newer hydrogen bonds (Ngarize et al., 2004). The intensity of the spectral peak for heated protein is seen to be depleting at 1318 cm-1. This is amide III band and it reflects towards alpha-helical structure in both proteins (ovalbumin and *beta*-lactoglobulin).

On making a sum up from the data, Proteins showed multiple beta sheet component in the range of 1680-1690 cm-1. This is the projection of protein aggregation and formation of hydrogen bonds. Heat treated proteins showed rise in beta-sheet formation for both proteins (ovalbumin and *beta* lactoglobulin). Although this result was not observed in high-pressure-treated samples. In the heated samples of both proteins elevation in the *beta*-sheet structure was observed with the depletion of alpha-helix structures (Ngarize et al., 2004). While the samples with high pressure treatment gave some changes in their secondary structure in different manner for each of the protein samples. Rise in random structure and decline in alpha-helical structure was seen for beta-lactoglobulin. Despite of this, ovalbumin gave an inflation in *beta*-turns with not notable change in alpha-helical structure and *beta*-sheets (Ngarize et al., 2004).

Effect of Heat and Pressure on beta lactoglobulin seen in Spectral band through FTIR:

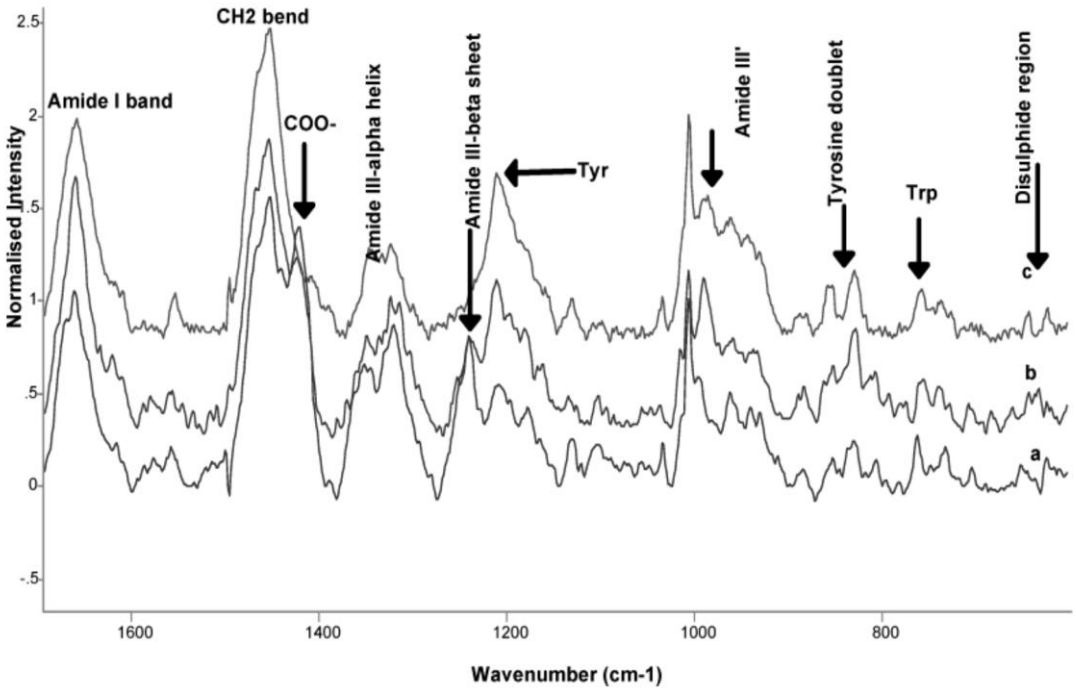


Figure 14 Spectrum of alpha lactoglobulin in three different sample form a) native, b) heated and c) high pressure treatment (600 MPa for 20 minute) (Ngarize et al., 2004).

Protein extraction is seen to be higher in samples with smaller particle size distribution. This can be explained as the surface area is enhanced for water and heat activity during treatment. The content of extracted protein fraction varies as per their molecular weight. As the complex molecules gets denaturation, association or disassociation to form smaller molecular fraction of protein undergoing processing. Breaking of disulphide bond and forming of newer sulphide bond may result in the yielding of insoluble fractionate protein.

Protein Fractional Band distribution of Maize through Electrophoresis:

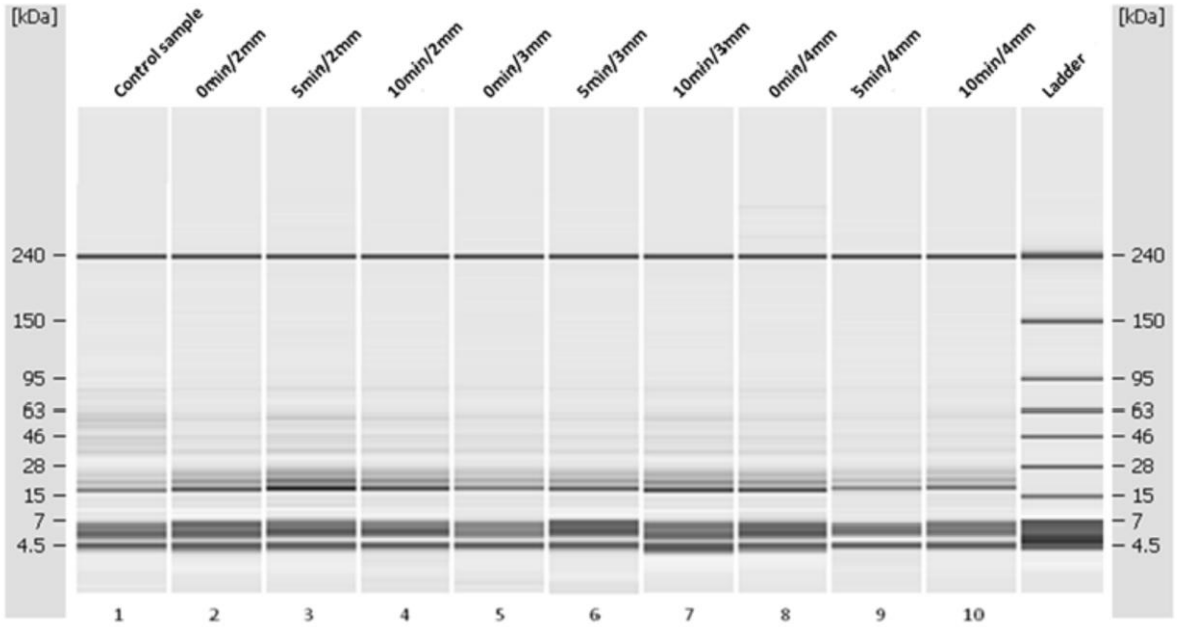


Figure 15 Picture showing the bands distribution of protein fraction on a gel image of maize (Čolović et al., 2013).

Looking over the bands of maize samples on the Gel image, the distribution of the bands over the image is near about same while the intensity of the bands is a bit different. This reflects that the extracted fractions as qualitatively equivalent but on quantitative scale it is different. Also, the difference in the quantitative scale of protein fraction is seen between control and heat treated (pelleted) samples. Higher concentration of protein fraction is seen with the smaller molecular weight (18 to 28 kDa) of protein.

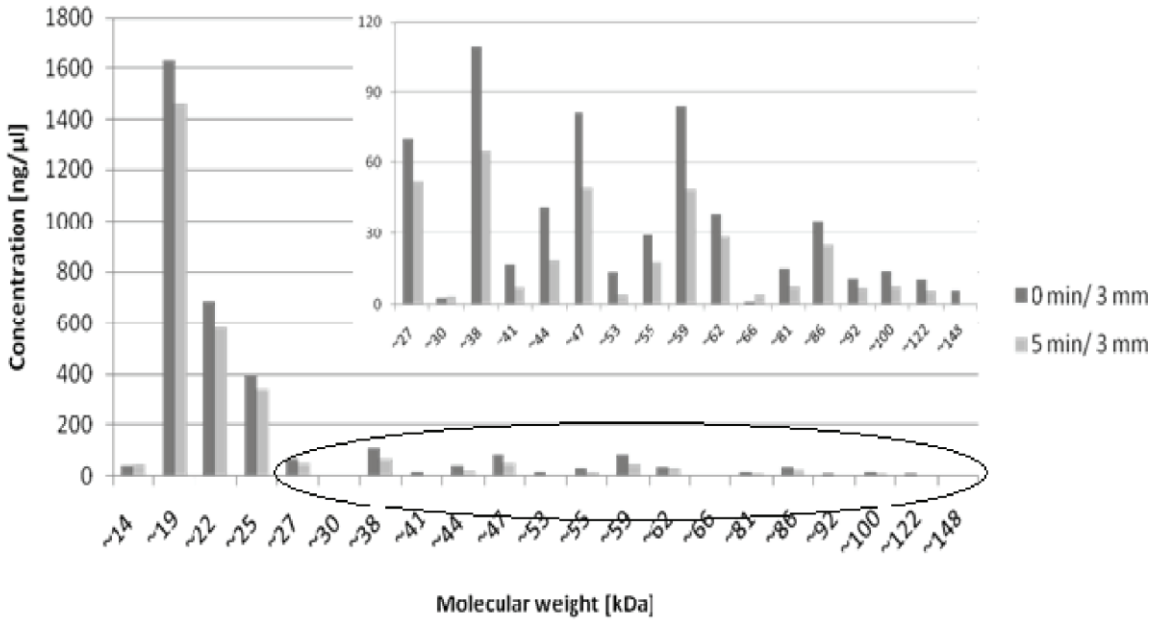


Figure 16 Distribution of protein fraction with respect to their molecular weight (Čolović et al., 2013).

When the concentration of the control sample and pelleted sample was compared there was threefold reduction (156.08 to 45.49 ng/µl) in pelleted sample concentration for 62.50 kDa of molecular weight (MW). Similarly, for 47.85 kDa of MW concentration reduction was about half (143.67 to 83.39 ng/µl). Looking over the protein fractions having smaller molecular weight, the concentration was on a rise in pelleted maize sample over the control sample. The result for 25.42, 22.40, and 18.70 kDa was (389.82 to 466.15 ng/µl), (640.47 to 811.22 ng/µl) and (1648.56 to 1951.93 ng/µl) respectively. Form this result we can conclude that the dissociation of the protein molecule in to smaller ones takes place during pelleting. The time assigned for Conditioning prior to pelleting have a noticeable effect on the protein denaturation in maize sample. As the retention time is increased the concentration of the protein fractions seems to get lowered with the exception for 14.30 kDa of MW as shown in figure.

Antinutrients (ANF):

Antinutrients are the compound present in the cereal grains and or legume seeds which reduce the digestibility of the major component of the food or feed system (Medic, Atkinson, & Hurburgh, 2014). It negatively inhibiting the growth of feeding animals. Soy protein is one of the major source for monogastric animals. Selection of soy is better in terms of poultry feed due balance in the amino acid profile. Methionine is the only known missing amino acid in soybean meal. Beside of these beneficial components soybean contains numerous amount of antinutrients. Some of the known antinutrients are Protease inhibitors (Kunitz trypsin inhibitors, Bowman-Brik Inhibitors approximately about 6 % of the soybean protein), Lectins, Saponins, Phytic acids (Medic et al., 2014). Some of the food legumes contains Tannins as antinutrients. Kunitz Trypsin inhibitors have seven cysteine subunits and is heat labile. Bowman-Brik inhibitors have two cysteine subunits and is heat stable.

Protease inhibitors act on the active site of enzyme activity (trypsin and chymotrypsin) and inhibit the protein digestion. In a study done on rats showed that this inhibition leads to hypertrophy of pancreas (Friedman & Brandon, 2001). Overcoming of this limitation is done by selection of the breed species. Along with this heat treatment is also one of the common measure to eliminate these antinutrients.

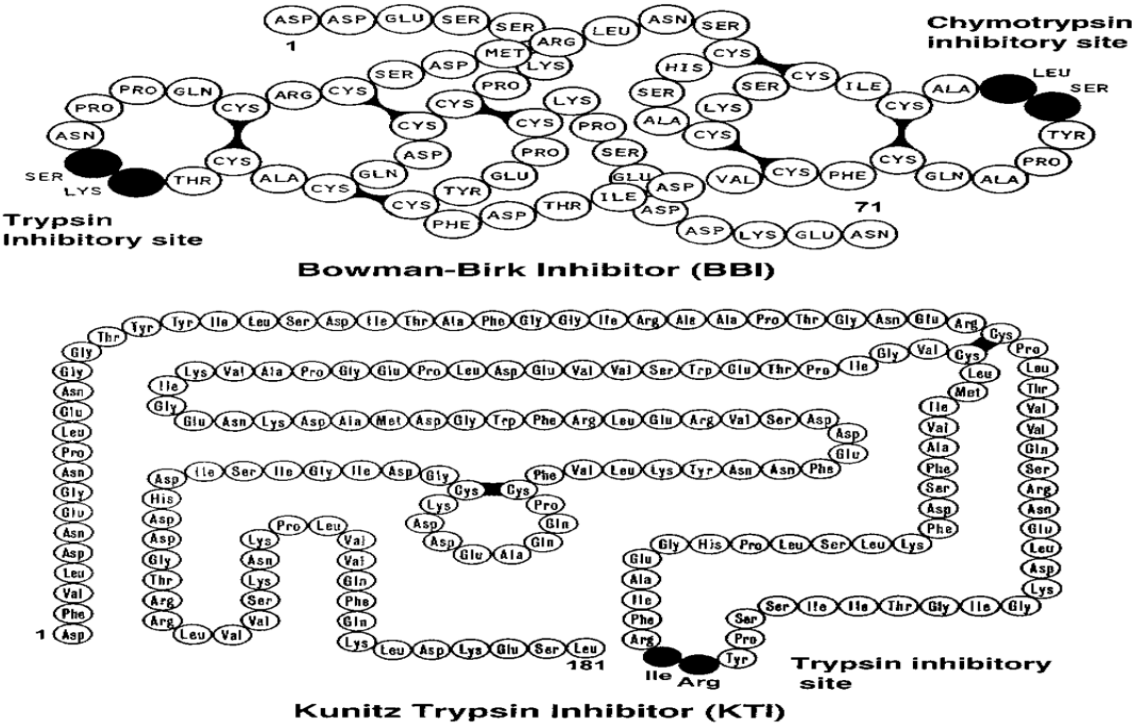


Figure 17 Protease Inhibitors showing active site for enzyme activity inhibition (Friedman & Brandon, 2001).

Heat treatment reduces the amount of antinutrients but there is a challenge that heat alone cannot eliminate this antinutrients (Friedman & Brandon, 2001). Also, loss of heat sensitive amino acids (Lysine, Cysteine and Methionine) is seen. These heat sensitive amino acids are prone to react with free carbonyl group of sugar in the feed leading towards maillard reaction. Antinutrients like Phytic acid and Oligosaccharides are heat stable molecule which cannot be eliminated by heat treatment. Phytic acid binds the metallic ions and minerals making a complex. This leads to the limitation of mineral (calcium, phosphorus, magnesium, copper, iron and zinc) uptake for animal growth. Use of enzymes (NSP enzymes and phytase) are beneficial in overcoming this type of antinutrients.

Maillard Reaction:

Maillard reaction is a non-enzymatic reaction which occurs when a reducing sugar reacts with a free amino group present in amino acid. It is also called browning reaction and is generally ongoing reaction at room temperature. Reactive amino group binds with the carbonyl group of reducing sugars like glucose or lactose. These reaction leads to the formation of Schiff base, also referred as Amadori compound (Dutson & Orcutt, 1984). Further on this compound undergo Amadori rearrangement to yield ketose amine, reductones, furfurals and other cyclic

compounds. Amadori compound and Schiff base passes more reactions to get melanoidins. Melanoidins are the final products responsible for the browning of product.

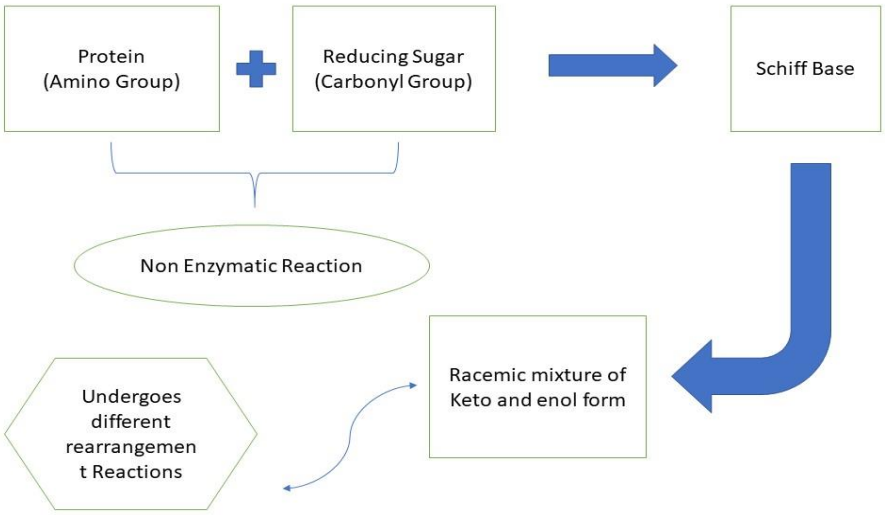


Figure 18 Non-enzymatic reaction of sugar and amine forming Schiff base.

Lysine considered as most reactive amino acid as it contains free amino group present at the epsilon carbon unit. It is highly prone to maillard reaction in free form as it contains two amino group to react with sugar during processing. Lysine gets converted in to fructoselysine which is the intermediate compound. Sometimes, crosslinking of the two different amino acids takes place. For example, alanine and lysine get crosslinked forming lysinoalanine and under different reaction pathway leads to formation of N-carboxymethyllysine (CML) and (5-hydroxymethyl)-2-furfural (HMF) (van Rooijen, Bosch, Wierenga, Hendriks, & van der Poel, 2014). These products are the cause to generate age related diseases in human and dogs. These derivatives do get absorbed poorly and show minimized biological activity. Maillard reaction depends upon numerous factors such as; pH, temperature, time, water activity, pressure during treatment (Ajandouz & Puigserver, 1999). This browning reaction can cause loss in nutritional quality of feed, also loss in palatability. The loss of sugar molecule is higher compared to protein molecule during this reaction, but loss of protein molecule is of greater concern for the production. Most often we do miss to recognize maillard reaction as we are looking for the change in colour for its prediction. But it should be always taken in consideration that change in colour and loss of amino acid during processing are two different things. Amino acids can get denatured before it shows any reflection as a colour change.

Different Amino groups of protein such as lysine, thiamine, folacin and gossypol are vulnerable to browning reaction. Intermediate products of Maillard reaction are N-(1-Deoxy-D-lactulosyl) lysine and N-(1-Deoxy-D-lactulosyl) lysine. These compounds could not get absorbed in mammals and avian as it cannot be deaminated in these species. Intestinal microflora can only unlock these compounds by going through deamination process.

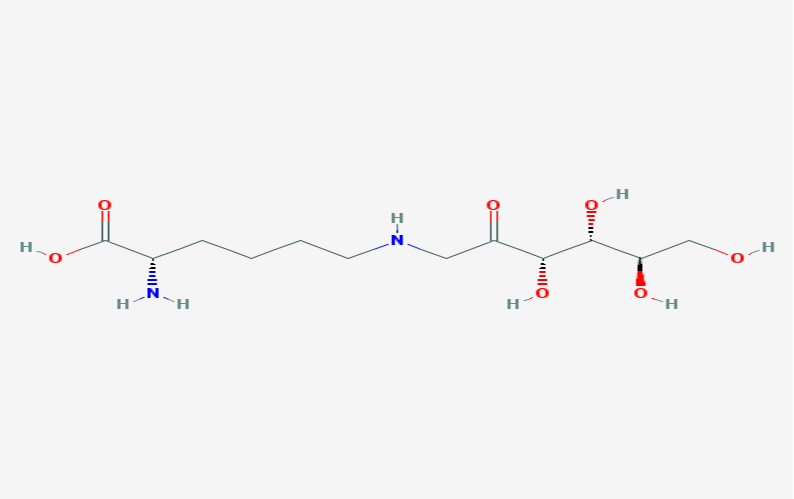


Figure 19 Structure of N-(1-Deoxy-D-fructosyl) lysine (Pubchem, 2006).

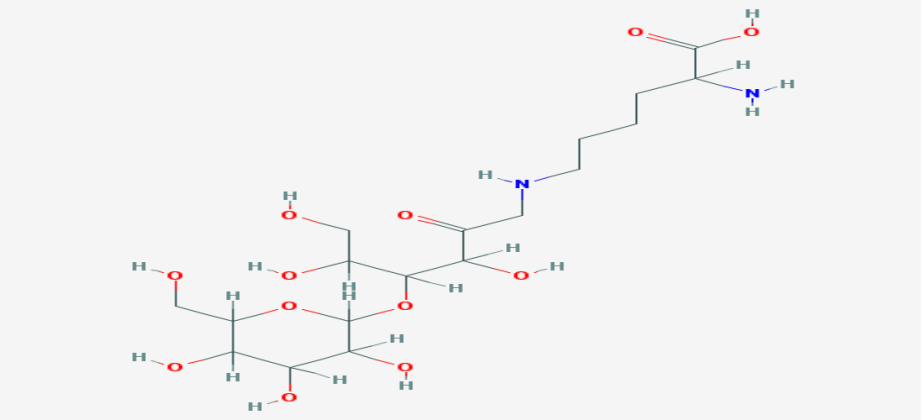


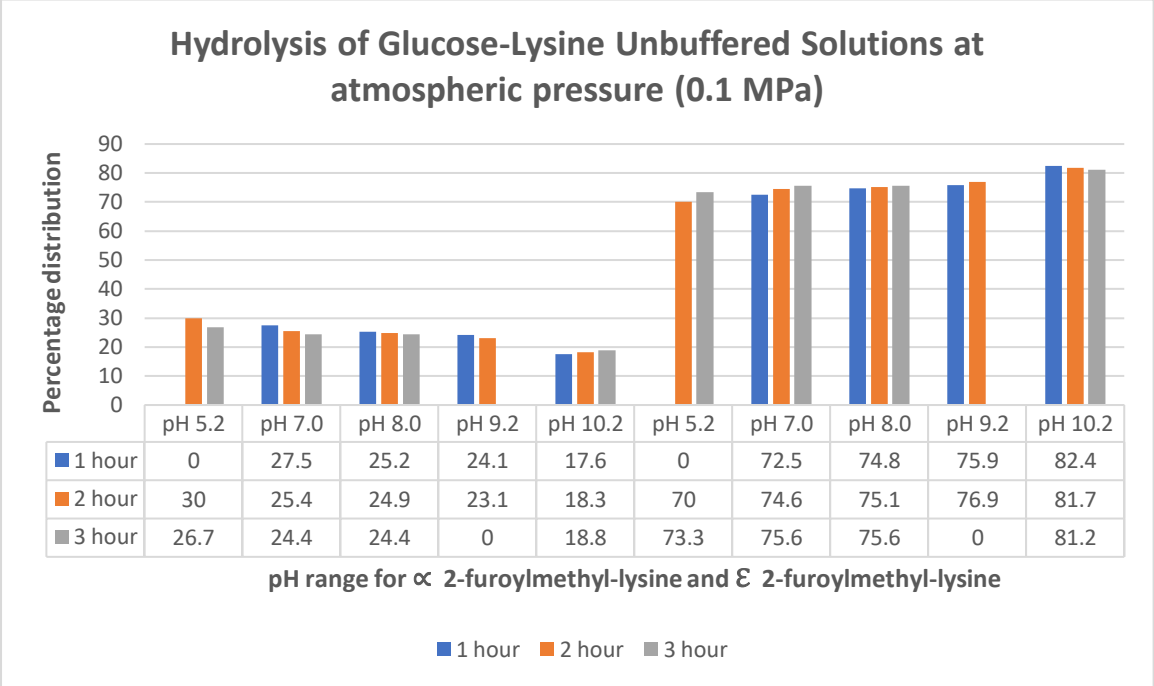
Figure 20 Structure of N-(1-Deoxy-D-lactulosyl) lysine (Pubchem, 2005).

Effect of Pressure on Maillard Reaction:

Use of high pressure from 100 to 1000MPa during processing in food and feed industry is increasing. Also, combination of pressure with heat may affect the extent of maillard reaction with change in colour, flavour, digestibility and functionality of macromolecules used during processing mainly protein and polysaccharides. Beside the detrimental effect of maillard reaction there are some beneficial role of this reaction such as gaining desirable flavour of foods

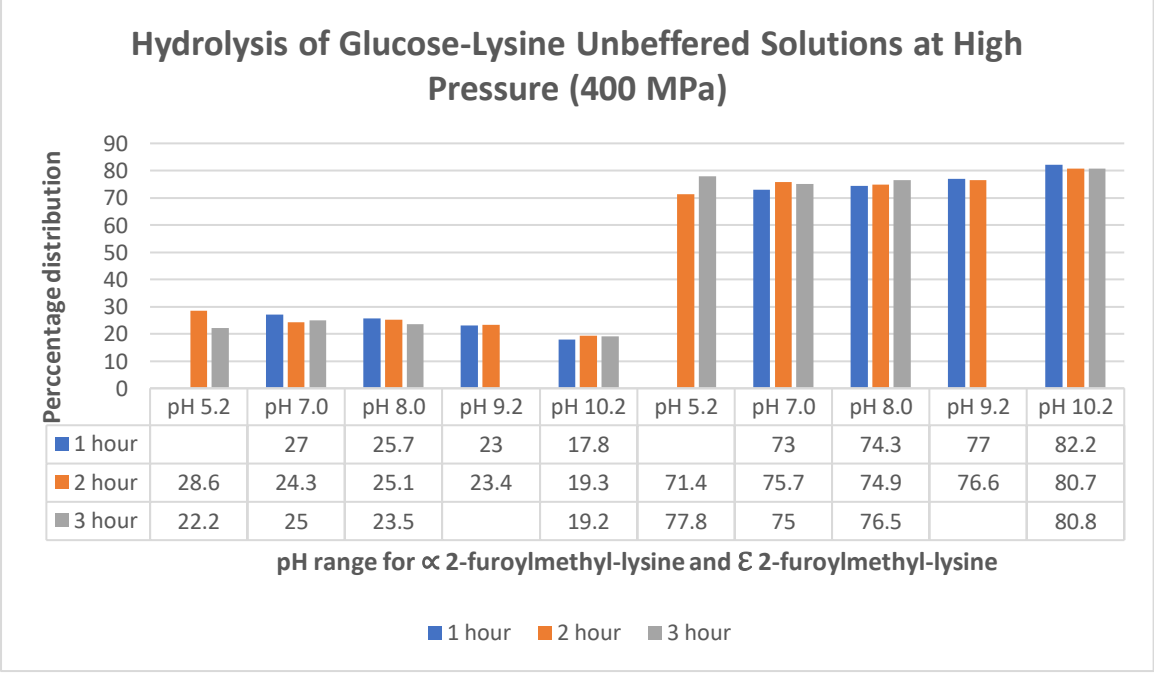
and feeds. Below it is being analysed the effect of pressure on the lysine and glucose in buffered and unbuffered solution with different PH range incubated at 60ºC temperature.

Effects of High Pressure on the Maillard Reaction on Glucose-Lysine Unbuffered Media:



	pH 5.2	pH 7.0	pH 8.0	pH 9.2	pH 10.2	pH 5.2	pH 7.0	pH 8.0	pH 9.2	pH 10.2
1 hour	0	27.5	25.2	24.1	17.6	0	72.5	74.8	75.9	82.4
2 hour	30	25.4	24.9	23.1	18.3	70	74.6	75.1	76.9	81.7
3 hour	26.7	24.4	24.4	0	18.8	73.3	75.6	75.6	0	81.2

Figure 21 Contents of 2-Furoylmethyl-lysine after Acid Hydrolysis of Glucose-Lysine Unbuffered Solutions (Moreno, Molina, Olano, & López-Fandiño, 2003).



	pH 5.2	pH 7.0	pH 8.0	pH 9.2	pH 10.2	pH 5.2	pH 7.0	pH 8.0	pH 9.2	pH 10.2
1 hour		27	25.7	23	17.8		73	74.3	77	82.2
2 hour	28.6	24.3	25.1	23.4	19.3	71.4	75.7	74.9	76.6	80.7
3 hour	22.2	25	23.5		19.2	77.8	75	76.5		80.8

Figure 22 Contents of 2-Furoylmethyl-lysine after Acid Hydrolysis of Glucose-Lysine Unbuffered Solutions (Moreno et al., 2003).

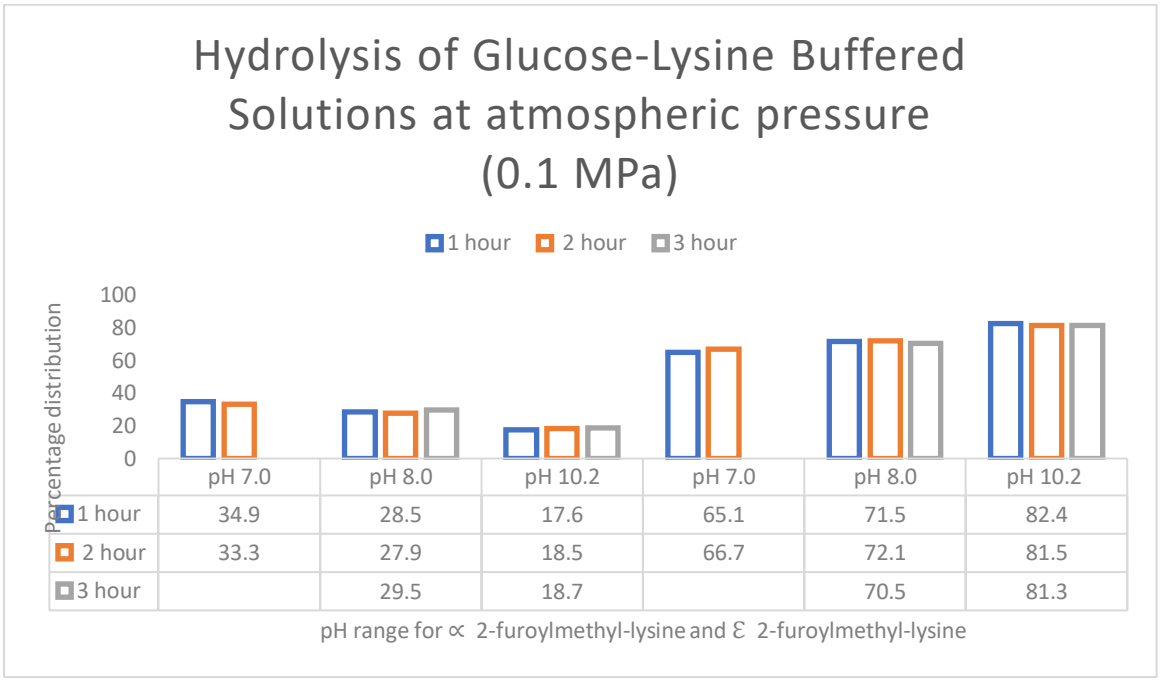
Above bar diagram depicts the percentage distribution of 2-furoylmethyl-lysine derivatives i.e. α and Ɛ i.e. heated in three distinct sample for a period of 1, 2, and 3 hours respectively. Two pressure system is used for observing the change due to induced pressure. One of them is placed in atmospheric pressure (0.1MPa) and the other one is high pressure (400MPa). In the first stage of study, solution used is an unbuffered one which is incubated at 60ºC. pH is distributed from slightly acidic to highly alkaline level, i.e. 5.2, 7.0, 8.0, 9.2 and 10.2 for the sample to be tested. The formation of Amadori Rearrangement Product (ARP) ascent markedly with rise in the alkanity of the solution. N-Ɛ-Amino derivatives were found to be in more abundant amount compared to that of N-α-amino derivatives. These differences between two derivatives seemed to be higher with the change in pH (glucose-lysine solutions). At pH 5.2, ~70% of Ɛ-2-furoylmethyl-lysine was observed while at pH 10.2 ~80% was detected. On regard to change in the pressure (400MPa), at different pH range from slightly acidic, neutral and slightly basic solution did not showed any significant difference. Although there was a notable rise in amount of 2-furoylmethyl-lysine at 1 hour in pressurized systems of pH 10.2 solution. Looking at 2 and 3 hours, there was a decrease. On the other side of unpressurized systems 2-furoylmethyl-lysine content gets increased continuously. From this it was considered that at pH 10.2, the initiation of the Maillard reaction along with the degradation of Amadori product takes at a faster rate (at higher atmospheric pressure of 400MPa than then that of 0.1MPa).(Moreno et al., 2003)

Effects of High Pressure on the Maillard Reaction on Glucose-Lysine Buffered Media:

Table Making a buffer solution by adding sodium phosphate buffer showed an enhanced formation of 2-furoylmethyllysine while in comparison with nonbuffered systems at pH 7.0 and 8.0. This rise in content is explained due to catalytic action of phosphate ion (anion) during Maillard reaction. As it is a known phenomenon that Maillard reaction can occur at normal temperature. In this buffer system initiation of reaction takes place rapidly at pH 7 under normal atmospheric pressure. The Amadori compound formed at PH 8 was similar under both normal and high pressure till 2 hours of incubation but formation of ARP declined at 400Mpa. At pH 10.2 in a buffered system the levels of 2-furoylmethyllysine was seen in lower value than in previous unbuffered systems (Moreno et al., 2003). This change can be explained on an assumption that, reaction was accelerated when buffered systems for glucose and lysine was used. This directed the reaction towards faster degradation of the intermediate compound formed during the reaction. At pH 7, increase in the reaction product was more significant at atmospheric pressure rather than at 400MPa, whereas at pH 8, the reaction product was found to be very similar in nature for both 0.1 and 400MPa. At pH 10.2, rate of reaction for buffered

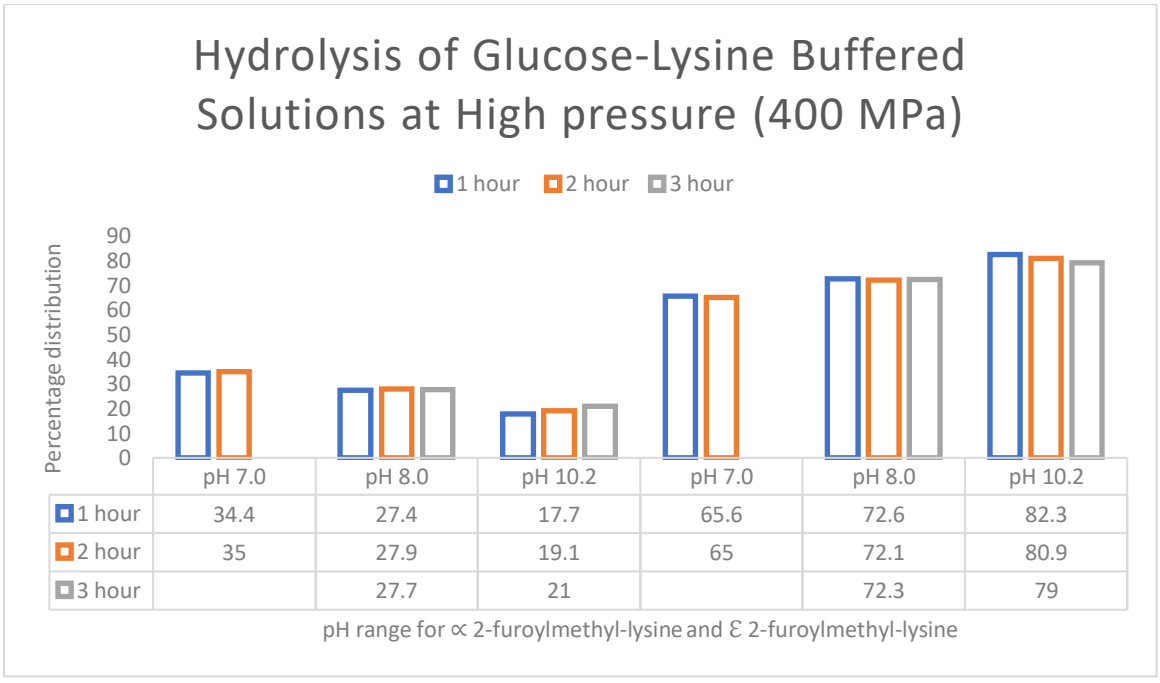
system seems to be in better level than comparing with unbuffered systems, especially in under pressure (Moreno et al., 2003).

Summing up from both tabulated data, it can be said that maillard reaction is highly prone to PH change and the nature of the medium used during the formulation. Reactivity of lysine to glucose increase in alkaline PH on both normal pressure and high pressure (400Mpa).



	pH 7.0	pH 8.0	pH 10.2	pH 7.0	pH 8.0	pH 10.2
1 hour	34.9	28.5	17.6	65.1	71.5	82.4
2 hour	33.3	27.9	18.5	66.7	72.1	81.5
3 hour		29.5	18.7		70.5	81.3

Figure 23 Contents of 2-Furoylmethyl-lysine after Acid Hydrolysis of Glucose-Lysine Buffered Solutions (Moreno et al., 2003).



	pH 7.0	pH 8.0	pH 10.2	pH 7.0	pH 8.0	pH 10.2
1 hour	34.4	27.4	17.7	65.6	72.6	82.3
2 hour	35	27.9	19.1	65	72.1	80.9
3 hour		27.7	21		72.3	79

Figure 24 Contents of 2-Furoylmethyl-lysine after Acid Hydrolysis of Glucose-Lysine Buffered Solutions (Moreno et al., 2003).

Effect of PH on Maillard Reaction:

Lots of study are been done on the nonenzymatic browning reaction. The change of colour(brown) in glucose and lysine solution at different PH range is seen due interaction between glucose and lysine molecule. The understanding of maillard reaction is a bit complexed due to caramelization of sugar.

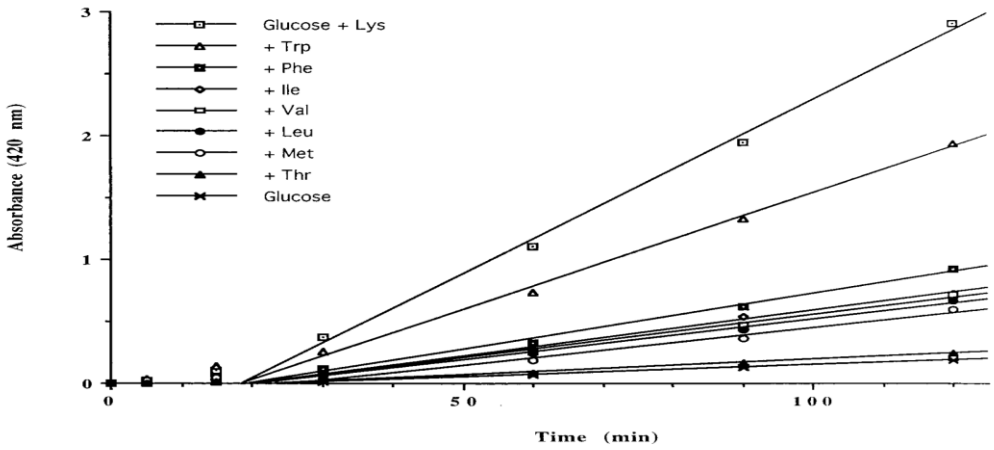


Figure 25 Colour development pattern in solution containing glucose alone or in combination with amino acid at 100ºC and at PH 7.5 with respect to time (Ajandouz & Puigserver, 1999).

Looking on the figure, it is seen that change in colour (browning of a glucose solution) is in increasing pattern as it is combined with the essential amino acids (threonine as an exception). On the other hand, none of the experimental amino acid on alone showed colour change with an exception of tryptophan. Lysine and tryptophan was seen to be more reactive compared to phenylalanine, isoleucine, valine as shown in table below. Leucine, methionine, and threonine showed lesser proneness to reaction. It is well known that Lysine is in the top list while Arginine in bottom list for non-enzymatic browning reaction. Different amino acids groups like acidic, SH-containing and hydroxyl may show negligible effect on the browning reaction. Longer lag time for the Methionine can be explained due to inhibitory effect of sulphur in the reaction. Complete absence of any undesirable effect before the lag time is noted in this reaction.

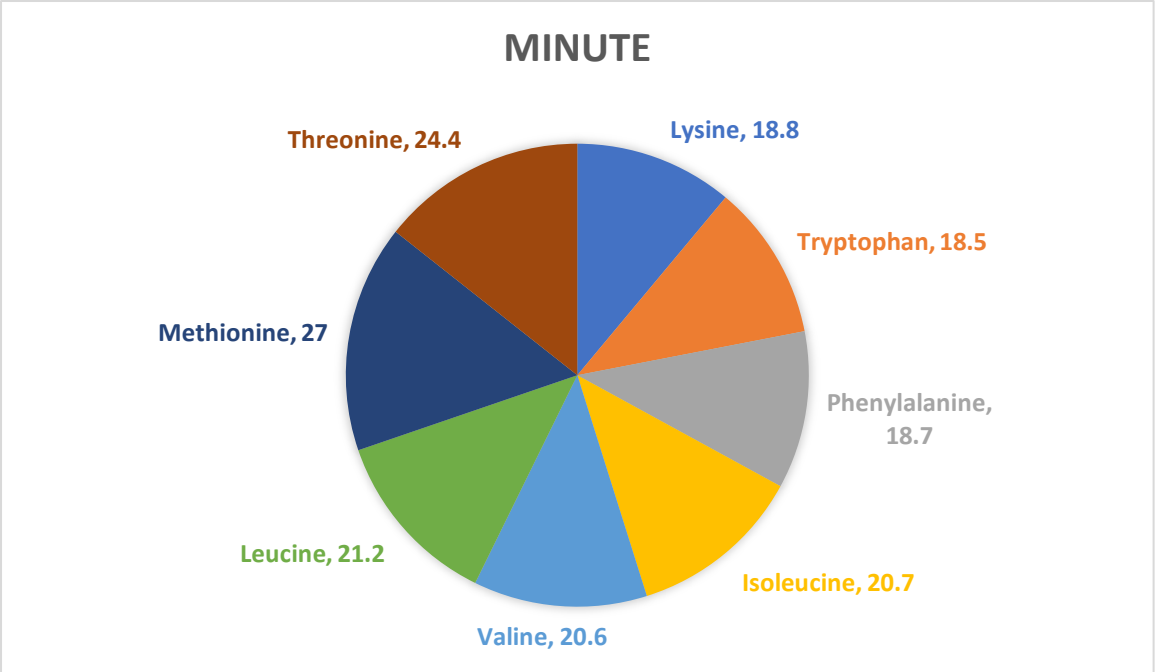


Figure 26 Lag time for browning reaction of solution containing glucose in combination of amino acids (Ajandouz & Puigserver, 1999).

Maillard reaction kinetics is difficult to understand and lesser study on this is done. As we can see from the above pie chart that each amino acid have their own lag time profile for showing the reactivity to the glucose molecule.

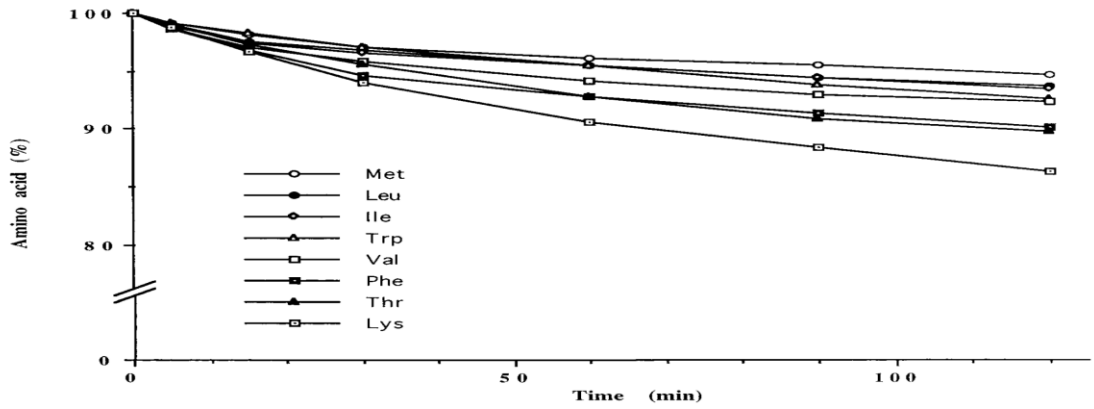


Figure 27 Loss of amino acid in combination of glucose with respect to time at 100ºC and at PH 7.5 (Ajandouz & Puigserver, 1999).

Discussion:

The demand for enhancing the quality of the pelleted feed especially the protein content in it, is gaining a height day by day. Treatment of protein in food industry have severe condition in which high pressure and temperature is used. But in context of pelleting we do use 60 to 90-degree C, this is milder condition of treatment been done. Structural changes in the protein during pelleting is not to that extent, mild denaturation is seen which help in binding of the molecule with starch present in the feed mash (M. Thomas & A. Van der Poel, 1996). This binding aid in developing texture of the pellet. Changes induced in the conformation of protein molecule projects the better digestion (proper uptake and utilization) of protein in the intestine of the animal.

Change in the content of alpha helix, beta sheet, beta turn and random coil is seen due to pelleting and pressure induced treatment (Ngarize et al., 2004). The change of ratio of beta sheet and alpha helix is found to be different as per type of protein being treated. Increase in conditioning time directly affect the fractionation of protein molecule (Čolović et al., 2013). Fragmentation of the protein molecule into smaller molecular weight is seen during pelleting and pressurized treatment. The amount of fraction for small molecular weight is found to be maximum (Čolović et al., 2013).

The degree of understanding on the chemical changes in past was a bit problematic as we do lack the efficient analytical technique to determine the exact change in the conformation of the polypeptide without altering its original (native) state. With the invention of newer technologies, the study on this subject is growing. Understanding the denaturation behaviour is becoming easier and the deeper insights of the protein fraction quality and quantity can be determined with greater efficiency.

Antinutrients present in the ingredients used for formulating pelleted feed is also been lowered with the aid of processing (Friedman & Brandon, 2001). Bacterial elimination is also seen to some extent of certain species of bacteria. But removal of spores of bacteria cannot be gained from pelleting. The limit of external parameter like heat and moisture for different ingredients which is responsible in initiating the Maillard reaction is gaining a better insight. As we know that Maillard reaction is ongoing at normal temperature as well. So, temperature used during pelleting is sufficient in destroying the amino acid content in the feed mash. This diminishing effect may have negative impact on the growth of animal. Lysine and Cysteine is highly prone to maillard reaction (Ajandouz & Puigserver, 1999). Lysine is highly reactive at alkaline pH

and the pressure system does not affect the rate of reactivity for lysine (Moreno et al., 2003). Maillard reaction is sometime confusing in nature with caramelization of sugar. The colour development seen in sugar caramelization is very less compared to the colour seen with amino acid conjunction reaction (Ajandouz & Puigserver, 1999).

Knowledge on the role of pepsin and other pancreatic enzymes in the digestion of protein and their activity on the denatured protein is been better understood. Enzyme activity is dependent upon the structure of protein either coming from same or distinct sources. In relation to the structure (mainly secondary structure), the solubility and digestibility of protein is greatly affected (Bai et al., 2016).

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