CHEMICAL AND ENZYMATIC MODIFICATION OF FOOD PROTEINS

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Introduction

Proteins are used abundantly in the food industries because of their nutritional value and techno functional properties such as emulsification, foaming, gelation, hydration and textural properties. These functional properties of proteins are related to their molecular structures and their ability to interact with other components of food matrix. Due to the inherent structural limitations in some food proteins, they lack the requisite physico-chemical properties to serve a function in foods. The effective utilization of proteins in food systems is dependent on tailoring the protein’s functional characteristics to meet the complex needs of the manufactured food products. Consequently, there have been number of ways to enhance the functionalities of many food proteins including physical, chemical and enzymatic methods. Modifications of functionality of food proteins increase their application as food ingredient.

A. Chemical modification of food proteins:

The primary structure of protein contains several reactive side chains. The physico-chemical properties of proteins can be changed and their functionality can be improved by chemically modifying the side chains. The various functional groups of proteins including amino, carboxyl, disulfide, imidazole, indole, phenolic, sulfahydryl, thioether and guanidine are the primary sites for derivatization. The nucleophilicities of the amino and sulfahydryl groups make them vulnerable to reaction. Consequently, reactions such as acylation, phosphorylation, esterification, glycation have been used to impart improved functional properties to the food proteins. Most of these chemical modifications are aimed at changing the net charge on the protein substituting ε amino groups, which in turn influence the solubility of the proteins. However, the principal utilization of chemically modified proteins in foods is limited because often the essential amino acid residues of the protein get derivatized. This may decrease the availability of attached amino acids, particularly lysine which is a primary target of electrophilic reagents. In addition, the derivative protein or its digestion products may be toxic. Nonetheless, chemical alteration of food proteins has been used with success to study the structure-function relationships (enzymatic function, biological function, physico-chemical and functional properties) to develop practical methods for modification of protein functionality and to link limiting amino acids covalently into deficient proteins.

Since proteins contain several reactive side chains, numerous chemical modifications can be achieved. However, only a few of these reactions may be suitable for modification of food proteins, which are described below:

1. Acylation of food proteins
Amino groups can be acylated by reacting them with several acid anhydrides. The most common acylating agents are acetic anhydride and succinic anhydride. Reaction of protein with acetic anhydride results in the elimination of positive charges of lysyl residues while succinic anhydrides results in replacement of positive charge with negative charge (Fig.1) (Chobert, J.M., 2010). This increase in negative charge of proteins results in drastic changes in the native protein conformation.

\[
P - \text{NH}_2 + \text{O} \quad \overset{\text{pH}\geq 7}{\longrightarrow} \quad P - \text{NH} - \text{CH}_3 + \text{CH}_3\text{COO}^- + \text{H}^+ \quad (1)
\]

\[
P - \text{NH}_2 + \text{H}_2\text{C} - \text{C} - \text{O} \quad \overset{\text{pH} \quad 7.5-9.5}{\longrightarrow} \quad P - \text{NH} - \text{C} - \text{C} - \text{COO}^- + \text{H}^+ \quad (2)
\]

Figure 1: Acylation reaction (Chobert, 2010)

1.1 Effect on functional properties:
Blocking of amino group by acyl residues changes the isoelectric point of the proteins to lower pH values, which results in a general shift of the solubility profile to move acid region and an increase in protein solubility in weak acidic, neutral and alkaline solutions. Succinylation of casein can result in improvement of solubility of casein. Acylation results in increase in water holding capacity of wheat gluten and peanut protein while that for sunflower and oat proteins decreased. The high affinity of succinylated proteins for water as well as adsorptivity at oil-water and water-air interfaces, impair their emulsification and foaming properties.

1.2 Effect on nutritional properties:
Acylation and succinylation reactions are irreversible. The digestion of acylated casein with pepsin/pancreatin give longer peptides than native casein. The succinyl-lysine isopeptide bond is resistant to cleavage by pancreatic digestive enzymes and poorly absorbed by the intestinal mucosa cells. Thus acylation reduces the nutritional value of proteins.

2.0 Phosphorylation of food proteins
Several natural food proteins including caseins, egg white albumin and egg white phosvitin are phosphoproteins. The introduction of phosphoryl residues increases the negative charge and hydration and changes the functional properties of proteins. Proteins can be phosphorylated by reacting them with phosphorus oxy chloride POCI₃ or sodium trimetaphosphate. Phosphorylation occurs mainly at the hydroxyl group of seryl and threonine
residues and at the amino group of lysyl residues (Fig. 2). Phosphorylation of amino groups results in addition of two negative charges for each positive charge eliminated by the modification.

\[
\text{Prot} \text{NH}_2 + \text{POCl}_3 \rightarrow \text{Prot} \text{NH} - \text{POCl}_2 + \text{H}^+ + \text{Cl}^-
\]

**Figure 2: Phosphorylation reaction (Damodaran, 1996)**

### 2.1 Effect on functional properties:

The functional properties of proteins can be improved or impaired by phosphorylation, depending upon the nature of protein used. Phosphorylation of zein resulted in improvement of water solubility between pH 2-9. Sodium tri-metaphosphate treatment of soya protein isolate shifted the isoelectric point to lower pH, with increase in solubility above pH 6.0. In case of casein and soybean glycinin, water solubility decreased on phosphorylation, due to protein cross-linking. The emulsifying activity of casein was decreased on phosphorylation while that for zein and soyprotein isolate increased. However the gelation properties of casein and gluten following treatment with POCl₃ improved due to cross-linking of these proteins.

### 2.2 Effect on nutritional properties:

The extent of digestion of phosphorylated casein after 24 h of both trypsin and α-chymotrypsin catalysed digestion was, however, the same for control casein and phosphorylated casein.

### 3.0 Glycosylation of food proteins:

Glycosylation is an interesting tool for improving food protein functionality. Introduction of saccharides into proteins make latter more hydrophilic. Amino groups can be reductively alkylated with aldehydes and ketones in the presence of reductants, such as sodium borohydride (NaBH₄) (Fig. 3)(Damodaran, 1996). In this case, the Schiff base formed by the reaction of the carboxyl group with the amino group is subsequently reduced by the reactant. Aliphatic aldehydes and ketones or reducing sugar can be used in this reaction. Reduction of the Schiff base prevents the progression of Maillard reaction, resulting in a glycosylation as the end product.

\[
R - \text{CHO} + \text{NH}_2 \xrightarrow{\text{pH} 9} R - \text{CH} = \text{N} \xrightarrow{\text{NaBH}_4} R - \text{CH}_2\text{HN} \\
R = \text{alkyl or polyl (sugar) group}
\]

**Figure 3: Glycosylation reaction (Damodaran, 1996)**
3.1 Effect on functional properties:

Glycosylation of β-lactoglobulin induced change in the secondary and tertiary structure and the hydrophobicity of the protein, the extent of which depended on the nature of attached sugar residues and on the degree of modification. The solubility of glycosylated casein increased in the range of their isoelectric points, unchanged in the neutral and alkaline conditions and decreased at low pH. Further an increase in the flexibility and unfolded state of glycosylated casein resulted in better foaming capacity and stability than native casein. Glycosylation of β-lactoglobulin not only result in the improvement of its surface properties but also provide protection against denaturation.

4.0 Deamidation of food proteins:

Deamidation is a hydrolytic reaction that converts glutamine and asparagines into glutamic acid and aspartic acid(Fig.4). It is catalysed by acids and bases (nucleophiles), and requires a water molecule. The general acid, HA, catalyses the reaction by protonating the amido-NH-leaving group of the Asn side chain. A general base (the conjugate base, A- or hydroxide ion) can attack the carbonyl carbon of the amido group or activate another nucleophile by abstraction of a proton for attack on the amide carbon. The transition state is inferred to be an oxyanion tetrahedral intermediate, whose stabilisation by proton donors increases the rate of the reaction. The order of acid- and base-catalysed steps varies with reaction conditions, particularly pH.

Figure 4: Deamidation reaction (Kumagai, 2012)

Glutamine and asparagines amide groups are usually hydrolysed by acid or base catalysis. However, this reaction is not specific, because it also causes splitting of peptide bonds. To minimize the hydrolysis of peptide bond, the denaturation of vegetable proteins has been performed in the form of cation exchange resins and common anions such as phosphate or bicarbonates as catalysts.

4.1 Effect on functional properties:

The deamidation of glutamine and asparagine residues in proteins results in liberation of carboxyl groups and thereby to an increase in negative charge and hydration. This is especially an important in case of wheat gluten, which has high content of glutamine (30% of its constituting amino acids), so even a low percentage of deamidation has large effects on charge and functionality. The deamidated proteins showed substantially improved solubility.
water binding capacity, foam expansion, emulsion viscosity as compared to their unmodified counterparts.

**B Enzymatic modification of food proteins**

Chemical modification is not very desirable for food applications because of the harsh reaction conditions, non-specific chemical reagents, and the difficulty to remove residual reagents from the final product. Modification of the molecular structure of food proteins with enzymes is an attractive way of improving the functional and nutritional properties of these proteins. Enzymes provide several advantages including fast reaction rates, mild conditions and high specificity. Proteases and transglutaminase are the most frequently used enzymes for modifying the polypeptide backbone of food proteins. Functional properties of proteins (solubility, gelation, and emulsification and foaming) are closely related to their size, structural conformation, and level and distribution of ionic charges. Limited proteolysis can enhance their functional properties over a wide pH range and processing conditions. These changes in the functional properties are attributed to changes in charge, hydrophobicity and molecular mass in going from protein to peptide mixtures.

**1.0 Limited enzymatic hydrolysis**

Endopeptidases cleave the peptide linkage between two adjacent amino acid residues in the primary sequence of a protein yielding two peptides. The specificity of an enzyme is a big factor influencing both the number and location of the peptide linkages that are hydrolysed. Proteolysis can proceed either sequentially or through the formation of intermediates that are further hydrolysed to smaller peptides often termed Zipper mechanism. One of the key parameters in protein hydrolysis is the degree of hydrolysis (DH). This is defined as the percentage of peptide bonds cleaved. The enzymatic hydrolysis of proteins depends on pH, temperature and the concentration of substrate and enzymes.

Hydrolysis of peptide bonds causes three distinct changes in proteins:

1. The NH$_3^+$ and COO$^-$ content of protein increases which increases its solubility.
2. The molecular weight of the protein/polypeptide decreases.
3. The globular structure of the protein is destroyed and the buried hydrophobic groups become exposed.

Enzymatically hydrolysed proteins are classified based on the molecular weight distribution of the resultant hydrolysate. Larger peptides (2-5 kDa) are mainly used as functional ingredients or in personal care products. Medium sized peptides (1-2 kDa) are used in clinical nutrition. Smaller peptides (<1 kDa) are used in infant food products required reduced allergenicity.

**1.1 Changes in functional properties:**

a) Solubility
Proteolytic modification has special importance for the improvement of solubility of proteins. This effect becomes significant even after very limited proteolysis. Zein, the maize protein that is highly insoluble at pH 2-5, exhibited good solubility (30-50%) at this pH range when only 1.9% of the peptide bonds were split by treatment with trypsin. Hydrolysis of casein to DH 2 and 6.7% with *staphylococcus aureus* V8 proteases increased the isoelectric solubility to 25 to 50%, respectively (Schwenke, 1997).

b) Emulsifying property
Emulsifying properties of proteins are sensitive to proteolytic modification. Limited hydrolysis (DH 2 and 6.7%) of casein decreased the emulsifying activity (EA) at all pH whereas the emulsion stability (ES) at DH=2% was higher than native casein. The EA of casein was reported to decrease with increasing net charge and with the decreasing hydrophobicity due to proteolysis. The beneficial effect of limited proteolysis of globular proteins on EA and ES may be due to exposure of buried hydrophobic groups, which may improve the hydrophobic–hydrophilic balance for better emulsification. The loss of hydrophobic peptides from the surface of the proteins may directly result in an increase of the surface hydrophobicity, thus favouring surface adsorption. The detrimental effect of excessive digestion is related to the loss of globular structure and optimum size of split peptides, resulting in formation of a thinner protein layer around the oil droplets and a loss of stable emulsion.

c) Foaming property
The excellent foaming properties of soybean protein hydrolysate made using acid aspartic protease from *mucor miehei* might be due to narrow molecular weight distribution of peptides produced by this enzyme. This enzyme is very specific and can hydrolyze very few of the peptide bonds in soy protein. At 0.5% DH, the soy protein hydrolysate made with this enzyme behaves like an egg white substitute with excellent foaming properties. Protamex hydrolysates of sodium caseinate (DH 0.5 and 1.0%) displayed increased from expansion at pH 2, 8 and 10 as compared to native casein.

d) Gelation
Hydrolysis was presumed to be detrimental to the gelling properties of protein because of the reduced hydrophobicity of hydrolysates. The increased net charge on the protein results in increased charge repulsion between peptides, decreasing their gelling ability. The loss of gelation ability of soy protein isolate is used to advantage in manufacture of soy protein hydrolysate that can be heat processed without changing their flow properties. Limited proteolysis of whey protein isolate exhibited gelation characteristic that were different from those of untreated whey protein isolate.

2 Progressive proteolysis
Liberation of biologically functional peptides
Until, recently, food proteins were regarded mainly as a source of amino acids essential for the proper functioning of the human body. Recent years have witnessed a growing interest in the use of biologically active peptides (BAP) in the production of functional foods. In addition to its basic function, every protein can play the role of a precursor of biologically active peptides, which are activated only after they have been released from the protein chain
by proteolytic enzymes. Biologically active peptides found in food proteins usually contain 2 to 9 amino acid residues. Some, however, may comprise even 20 or more amino acid residues. For example, caseinomacroppeptide contains 64 amino acid residues and shows many types of activity. Biologically active peptides may be released from their protein precursors in three ways: during (a) enzymatic hydrolysis by digestive enzymes; (b) fermentation processes involving proteolytic starter cultures; and (c) proteolysis involving enzymes of animal and plant origin or microbial enzymes.

In most cases, these protein hydrolysates and peptides have demonstrated better bioactivity compared to their parent proteins, and this shows that hydrolysis of peptide bonds is important in liberating the potent peptides. Several factors affect the bioactive properties of the peptides including the enzymes used for hydrolysis, processing conditions, and the size of the resulting peptides, which greatly affects their absorption across the enterocytes and bioavailability in target tissues. Most reported BAPs are produced by in vitro enzymatic hydrolysis or fermentation. After selecting an appropriate food protein, enzymatic hydrolysis is performed using single or multiple specific or nonspecific proteases to release peptides of interest.

Upon oral administration, BAP may affect the major body systems, namely, the cardiovascular, digestive, immune and nervous systems, depending on their amino acid sequence. These beneficial health effects may be attributed to numerous known peptide sequences exhibiting antimicrobial, antioxidative, antithrombotic, antihypertensive and immunomodulatory activities. Once these peptides released from parent proteins, they may act in the body as regulatory compounds with a hormone-like activity.

3 Enzymatic cross-linkage reaction

Transglutaminase (TG EC 2.3.2.13, glutaminyl-peptide, amine-γ-glutamyl transferase,) catalyzes an acyl transfer reaction between the γ-carboxyamine group of a protein-bound glutamyl residue and a primary amino group of various substrates including the ε–amino group of lysine or lysyl residues in proteins, resulting in polymerization or amine incorporation (Fig. 5, 6) (Buchert, et al., 2010). The crosslink formed is called a ε–(γ-glutamyl)-lysine isopeptide bond (Kumagai, 2012). In the absence of amines, water serves as acyl acceptor leading to the conversion of glutamines to glutamic acid (deamidation) but is more frequently used for attachment of amino acids and especially cross-linking (reactions with primary amines). Cross-linking results in the formation of bonds between glutaminyl and lysyl residues from the same protein molecule (intramolecular) or from two separate molecules (intermolecular). By this intermolecular cross-linking covalent interactions between proteins are brought about resulting in, for instance, enhanced gel strength or network formation in milk, meat and bakery products.

3.1 Applications of enzymatic cross – linking of food proteins:

a) Cereal products:

Cross linking of cereal proteins has major potential in modification of technological and sensory properties of cereal products. TG mediated protein crosslink results in tailoring of rheological properties of gluten by decreasing dough extensibility,
increased water absorption and hinder the growth of air bubbles in the dough, thereby decreasing volume of bread. In the preparation of pasta and noodles, cross linking of gluten causes an increase in resistance for thermal processing.

b) Milk products:
Creation of inter-/ or intra molecular covalent bonds by TG cross-linking of milk proteins results in modification of textural and water binding properties of milk products especially, in low fat or protein products with acceptable texture.. In the production of fermented milk product such as yogurt, the introduction of covalent bond by enzymatic cross linking into gel network results in increase in gel firmness and sensory properties.

c) Meat products:
Enzymatic cross linking of the myofibrillar protein myosin improves the texture and water holding properties of meat or fish products and add value to meat and fish of poorer quality. In addition to restructuring, TG is successfully exploited in improving textural properties of heated meat products such as hams and sausages and enhancing surimi (fish paste) gelation and textural properties.

Figure 5: Enzymatic crosslinkage reaction (Kumagai, 2012)

Figure 6: Transglutaminase reactions (Buchert, et al., 2010)
Conclusion:

Proteins are fundamentally important as nutrients but now they are seen to be much more. Through their unique functional properties, they can become key ingredients that determine many parameters of food quality. However, most proteins still have scope for further improvement in their physical and functional properties. Chemical modification is not very desirable for food applications because of the harsh reaction conditions, non-specific chemical reagents, and the difficulty to remove residual reagents from the final product. Enzymes have long been used for modifying food proteins and their use is many times more acceptable to improve the physical and functional properties of proteins.

References: