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Review

Applications of protein crosslinking in food products

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Abstract

In the past few years, researchers have focused on improving the functional properties and qualities of food products. To this end, they have used crosslinking for enhancing the functional properties of proteins in the food products. Enzymatic or non-enzymatic crosslinking can be used to modify food proteins. Protein crosslinking is efficient in generating novel textures and developing product formulations, while also maintaining the desired texture and mouthfeel of food products. Enzymatic treatments using laccases, transglutaminases, peroxidases, and tyrosinases could help in designing meat replacement products, and developing non-dairy yoghurt and cheeses with good consistency. However, these catalytic mechanisms are accompanied by many technical issues that need to be overcome while developing complex food matrices.

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Introduction

Protein is a highly complex polymer consisting of twenty different amino acids which are made up of an α-carbon atom, covalently attached to a hydrogen atom, an amino group, a carboxyl group, and a side-chain R group. Proteins are an essential nutrient, and play a vital role as they affect the physical characteristics of food products. The different amino acid sequences and compositions alter the conformation, structure, and functionality of the protein molecules (Ozdal *et al.*, 2013). Population expansion has led to increased food consumption which has caused a global food shortage. To resolve this issue, researchers have expressed a higher interest in investigating the applications of food proteins, which is expected to increase 2-fold by 2050 (Isaschar-Ovdat and Fishman, 2018). Reports have shown that animal protein consumption increased by 225% (between 1961 - 2007) in Asia. This was attributed to the fact that Asians have started shifting from plant-based diet to animal-based diet, which is more popular in developed countries (Boland *et al.*, 2013).

In an age of increased pressure and demand to meet consumers' diverse food needs, the understanding of functional changes in proteins during processing is essential. Majority of food proteins display very good gelling, emulsifying, foaming, and thickening properties. Hence, the use of these protein molecules can improve the stability, texture, appearance, and mouthfeel of various food products (Jaberian *et al.*, 2013). Food manufacturers often modulate the functional properties of food proteins by altering the nature and number of crosslinks during food processing. Crosslinking often results in principal changes in the chemical, functional, and nutritional qualities of food proteins.

Protein crosslinking is the linking of amino acid residues in the protein polypeptide chain with the help of intra- or inter-molecular covalent bonds, which leads to the development of various kinds of linkages. Crosslink can be created within a single polypeptide chain, multiple subunits, single-chain protein as a part of a multiprotein complex, or multi-subunit protein. Also, protein-protein interactions can take place due to inter-subunit and remote or

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unrelated multiprotein complex crosslinks (Kluger and Alagic, 2004).

Crosslinking in food proteins can be divided into three categories which are natural, physical, and enzymatic/chemical (McKerchar *et al.*, 2019). Naturally, crosslinking occurs in food products even before they are processed. Physically, crosslinking is carried out in the food products by using some physical processes such as high-pressure, mechanical, cooling, or heating treatments. These physical changes are safe, and can be carried out along with other modification processes. Chemically, crosslinking involves the use of chemical reagents such as glutaraldehyde to alter the molecular properties of food proteins. These processes are harmful and cause toxicity. For this reason, enzymatic crosslinks using commercial enzymes such as laccases, peroxidases, and transglutaminases are attracting attention due to their high biodegradability, specificity, yield, efficiency, and catalytic rate (Li *et al.*, 2020).

Researchers have developed various commercial enzymes which display excellent functional properties such as higher resistance to salt, temperature or pH changes, reaction rate, and high substrate utilisation. Furthermore, enzymes are particularly suitable for food applications due to the stability in mild conditions. Crosslinking is attributed either to the direct enzymatic catalysis of the crosslinks, or indirectly due to enzymatic generation of the crosslinking agents. This leads to the oxidation of the reactive structures which results in a crosslink formation. Thus, enzymes can be effectively used individually or in combination for improving food qualities through protein crosslinking (Hou *et al.*, 2015).

In the present review, different types of protein crosslinking occurring in food matrices were discussed. The present review highlighted the applications of protein crosslinking to improve the properties of food products. The impacts of protein crosslinking on consumers were also discussed.

Non-enzymatic crosslinking in food proteins

Natural crosslinking

Natural crosslinking in food products help in maintaining the native 3-D structure, and controlling the flexibility level of the polypeptide chains. These crosslinks can be altered based on the shape and function of the proteins present in the complex food matrices. It is noted that the protein crosslinking is impacted by two factors, *i.e.*, the accessibility to the linked amino acid residues, and the presence of steric hindrances and structural proximity between the amino acids (Visschers and de Jongh, 2005). However, very few studies investigated the disulphide crosslinks between the amino acids. Disulphide crosslinking affects protein functionality, emulsifying capability, dough properties, and thermo-rheological properties. Besides, food proteins with disulphide bonds have better heat stability than proteins with no disulphide bonds (Buchert *et al.*, 2010).

Physical crosslinking

Physical crosslinking results from non-covalent bonds such as ionic interactions, hydrogen bonds, and hydrophobic interactions that occur during processing (Hennink and van Nostrum, 2012). Ionic crosslinking is often used in the formation of cold-set emulsion gels. In this method, a protein solution is heated above the thermal denaturation temperature of the globular protein under conditions that prevent protein aggregation. The conditions involve pH and ionic strength that result in a strong electrostatic repulsion between protein molecules. Then, crosslinking of the unfolded proteins take place due to a change in solution conditions or the addition of a crosslinking agent that will reduce electrostatic repulsion. As a result, the protein molecules associate with each other to form a gel network that increases the volume of the system (Liang *et al.*, 2020). Based on previous studies, cold-set gels can be formed via acidification of protein solutions using monovalent cations such as Na^+ or by adding divalent cations, for example, Mg^{2+} (Zheng *et al.*, 2019). These approaches result in the formation of physical crosslinks between protein molecules that improve the mechanical properties of gels.

Besides, covalent or non-covalent bonds such as hydrogen bonding and hydrophobic interaction (Figure 1) lead to the formation of a crosslink between proteins and polyphenols (You *et al.*, 2014). However, food manufacturers favour the conjugates which are formed with the help of covalent bonds due to their long-lasting interactions and strength, which show higher stability (Liu *et al.*, 2017). It has been reported that protein-polyphenol conjugates are developed covalently with the help of free-radical grafting or alkaline reactions (Gu *et al.*, 2017). The polyphenols are affected by the oxidation reaction, which occurs if molecular oxygen is present under

alkaline conditions. This reaction generates semi-quinone radicals that rearrange themselves to form quinones. The intermediate products react with the nucleophilic residues such as cysteine and lysine present in the polypeptide side chains. This yields a covalent crosslink, *i.e.*, C-S or C-N bonds, between the polyphenol and protein molecules (Quan *et al.*, 2019). In a free-radical grafting process, the hydrogen peroxide and ascorbic acid molecules act as the initiator system or redox pair. The amino acids present in the polypeptide side chains are oxidised by the hydroxyl radicals that are generated due to the chemical reaction between the redox molecular pair. Thereafter, these amino acids form covalent bonds with the polyphenols to yield a crosslinked molecule via the protein-polyphenol conjugate. The crosslinks between the proteins and phenolic compounds alter the physicochemical characteristics of proteins such as thermal stability, solubility, and digestibility (Ozdal *et al.*, 2013).

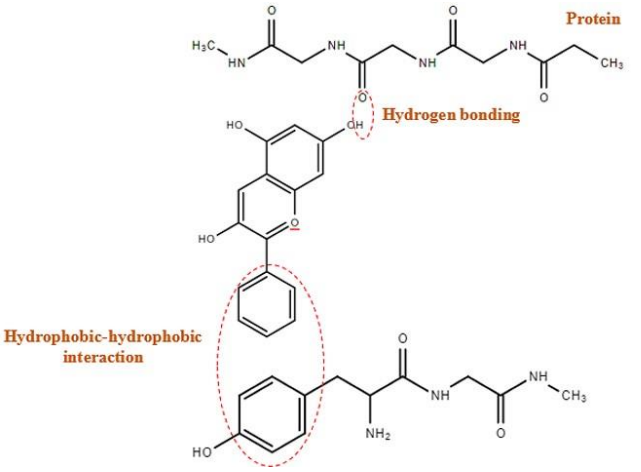

Figure 1. Protein crosslinking via hydrogen bonding and hydrophobic-hydrophobic interaction.

Furthermore, protein crosslinking could occur via the Maillard reaction that is also known as non-enzymatic glycation. This chemical reaction alters the flavours, colours, textures, and aroma related to browning of food items (Bastos and Gugliucci, 2015). During the primary stages of the Maillard reaction, the condensation reaction occurring between the amine groups (in protein molecules) and carbonyl groups (in reducing sugar molecules) yields a few Amadori products. Furthermore, during the intermediary and advanced steps, the formation of reactive intermediates such as sugar-derived dicarbonyl compounds was noted, which yielded a variety of polymerised compounds (Buchert *et al.*, 2010). The formation of covalent bonds between the protein molecules due to the Maillard reaction decreases the solubility of the protein molecules, which changes the water-holding capacity and rheological properties of the gel (Jumnongpon *et al.*, 2012). Though the Maillard reaction has garnered a lot of research interest, the details regarding the chemical reaction which led to protein crosslinking remain unclear.

Chemical crosslinking

Proteins can be crosslinked by introducing chemical crosslinking reagents that manipulate the properties of foods. Chemical crosslinker molecules are defined as bifunctional chemical compounds which contain some specific reactive moieties that represent the amine, carbohydrate, or sulfhydryl functional groups. A few chemical reagents form crosslinks with the amino acids, which leads to the formation of structurally-defined covalent bonds (Tinnefeld *et al.*, 2014). Glutaraldehyde is by far the most widely used chemical crosslinker owing to its high efficiency to react with protein and stabilise collagenous materials. Crosslinking between protein and glutaraldehyde occurs due to covalent inter- and intra-molecular bonding between aldehyde groups of glutaraldehyde and free amino groups of lysine or hydroxylysine residues of polypeptide chains (Fan *et al.*, 2018).

Currently, chemical crosslinking reagents cannot be used in food products. However, it is seen that these reagents behave as a vital tool for defining the presence of crosslinks in the food matrices that can alter the functional properties of the food products. Many researchers have investigated the interactions taking place in small protein subunits, larger assemblies, protein networks, and even large proteomes by using the chemical crosslinking step coupled to the mass spectrometry technique (Holding, 2015).

Enzymatic crosslinking

Enzymes are capable to crosslink proteins *in vivo* leading to the utilisation for diverse applications *in vitro*. Two types of crosslinking reactions can be differentiated based on the enzymatic mechanisms, which are direct covalent bonding catalysed by transferases, such as transglutaminase via protein-enzyme-thioester intermediates, and enzyme-mediated covalent bonding via reactive species that are enzymatically generated by oxidoreductases including laccase, tyrosinase, and peroxidase, which

subsequently react with proteins to produce protein networks. The enzymes discussed herein, and the proposed underlying crosslinking reactions are summarised in Table 1 and Figures 2 to 5, respectively.

Table 1. Summary of enzyme-mediated crosslinking in food proteins.

Enzyme	Crosslinking mechanism	Application	Effect	Reference
Transglutaminase (EC 2.3.2.13)	Acyl-transfer reaction	Protein network formation in chicken myofibrillar protein; gel stabilisation in rabbit meat; bread making; noodle processing	Improved texture; enhanced cooking yield; improved consistency; improved shelf-life; increased tensile strength	Canto *et al.* (2014), Li *et al.* (2017), Santhi *et al.* (2017), Gharibzahedi *et al.* (2019)
Laccase (EC 1.10.3.2)	Radical formation	Protein network formation in cheese; bread making	Enhanced antioxidant activity; increased yield; reduced hardness and chewiness; increased elastic and viscous modulus	Manhivi *et al.* (2018), Loi *et al.* (2018; 2020)
Tyrosinase (EC 1.14.18.1)	Quinone formation	Protein network formation; emulsion stabilisation	Improved distribution of droplet size; improved rheological behaviour	Isaschar-Ovdat *et al.* (2016)
Peroxidase (EC 1.11.1.x)	Radical formation	Protein network formation	Increased gel strength; increased storage modulus	Gui *et al.* (2020)

Transglutaminase

Many researchers regarded proteases as a major protein-modifying enzyme till the 1970s. However, studies conducted on the transglutaminase enzymes (EC 2.3.2.13, TG) (also called glutaminyl-peptide-amine γ-glutamyl transferases), showed that these enzymes were involved in protein crosslinking. This increased the scope of protein modification technologies (Miwa, 2020). Cytoplasmic TG2 was the first transglutaminase enzyme that was detected in the liver samples of guinea pigs in 1957 (Sarkar *et al.*, 1957). Thereafter, eight other kinds of TG enzymes were detected in different samples collected from plants, fish, and mammals. In 1987, researchers isolated the microbial TG enzymes after screening soil samples that were collected from different regions in Japan. The microbial TG was isolated from an actinomycetes species, *i.e., Streptomyces mobaraensis* (formerly known as *Streptoverticillium mobaraense* strain S-8112). Following enzyme characterisation, the researchers noted that the SH group present in the cysteine residue of the microbial TG enzyme acted as the primary enzymatic centre. They then isolated the 331-amino-acid polypeptide chain, having a molecular mass of 37863 Da from the S-8112 strain (Washizu *et al.*, 1994).

The classification of the TG enzymes showed that they belonged to the acyltransferase family, which formed a covalent crosslinking via their intra- or inter-molecular ε-(γglutamine)-lysine isopeptide bond between the γ-carboxamide group present in the peptide or protein-bound Gln residues and the ε-amino group present in the protein-bound Lys residue (Figure 2A). This enzymatic reaction involved the acyl-transfer reaction, wherein Gln was the acyl donor while Lys acted as an acyl acceptor. It was observed that the TG enzyme could catalyse the crosslinking between Lys and Gln, which was influenced by its side-chain accessibility that was dependent on the protein's structural conformation (Gharibzahedi and Chronakis, 2018a). Furthermore, if any amine substrate such as Lys is unavailable for the reaction, the TG enzyme could catalyse the deamination of the glutamyl residues after using water as the acyl acceptor (Figure 2B). The deamination converts glutamine residues to glutamic acid residues, thus resulting in the production of ammonia. In addition to these two reactions, TG could also catalyse the incorporation of other primary amines into glutamine (Gharibzahedi *et al.*, 2018b) (Figure 2C).

TG has garnered a lot of industrial success due to various reasons such as the detection of microbial TG enzyme, which is acquired in a large quantity, and possesses many enzymatic properties that offer advantages during food processing. Additionally, the

development of effective food processing applications has improved TG activity. Significant alterations in protein molecules present in food matrices occur due to the crosslinking induced by TG, thus resulting in better texture, stability, gelation, emulsifying potential, and water binding capacity without affecting the sensory or nutritional quality. Previous studies reported that the addition of TG to the food product formulation improved nutritive values by increasing the bioaccessibility and bioavailability of essential amino acids (Gharibzahedi *et al.*, 2018c).

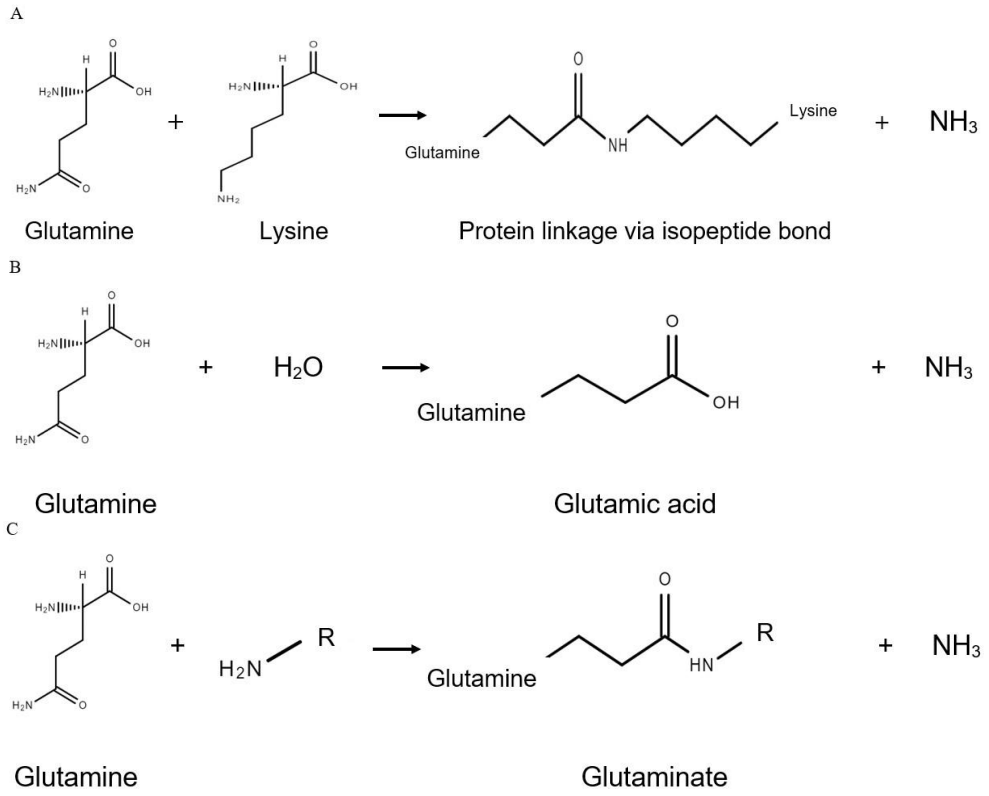

Figure 2. Transglutaminase-catalysed reaction; **(A)** crosslinking between glutamine and lysine resulting in isopeptide bond in protein, **(B)** deamination of glutamine using water as acyl acceptor, and **(C)** acyl transfer reaction.

Laccase

Laccase, which is a polyphenol oxygen oxidoreductase (EC 1.10.3.2) enzyme, was isolated more than 100 years ago. It exists in insects, plants, fungi, and bacteria as a monomeric protein with a molecular size that ranged from 40 to 100 kDa. Generally, laccases are isolated from fungal species since every lignified fungal species is known to produce the laccase enzyme (Senthivelan *et al.*, 2016). Furthermore, fungal laccases are exocellular enzymes that can be easily purified as compared to bacterial or plant-based laccases. Laccases catalyse any substrate which possesses a *p*-bisphenol structure such as aliphatic, aryl diamine, aniline, hydroxy indole, phenol, methoxy phenol, polyphenol, and benzenethiol (Bugg *et al.*, 2011).

These enzymes belong to the multinuclear copper oxidase family, and contain three cupredoxin-like domains which are used for their catalytic activity. Each member in this family possesses four Cu atoms that are arranged into the trinuclear or mononuclear centre at their active site (Martins *et al.*, 2015). These Cu atoms are categorised into three types depending on their varying spectral absorption patterns that are determined using specific spectroscopic methods. Type 1 site (T1) refers to the Cu atoms which act as the major electron acceptor, that is detected at 600 nm and appear blue. Type 2 site (T2) cannot be detected using any absorption spectrum. However, it activates the molecular oxygen species. Type 3 site (T3) is called the coupled binuclear because of the presence of two Cu atoms, and can be detected at 330 nm. T3 is responsible for oxygen uptake (Jones and Solomon, 2015).

Laccases catalyse the reaction between the oxygen atom and electron-rich substrate. In this reaction, the electrons are transferred to the trinuclear cluster by the His-Cys-His tripeptide which consists

of T2 and T3, after a single-electron oxidation step. The completely reduced laccase molecule reacts with the oxygen molecules via the electron transfer procedure to generate a water molecule, before returning to its primary state. A half mole of oxygen is required for every oxidation cycle. The oxidation product is an unstable, aromatic, and reactive radical which attacks the protein molecules (Wang *et al.*, 2018a), thereby leading to a crosslinking with the help of the Tyr (tyrosine) residues (Figure 3). The free radical that is generated undergoes oxidative coupling, wherein the molecules can homogenously or heterogeneously couple to yield polymers or dimers (Pezzella *et al.*, 2015). It was noted that the catalytic activity of laccase is associated with the redox potential that is dependent on ligand molecules for the T1 Cu site and amino acid residue (Castrovilli *et al.*, 2019). Eq. 1 presents the laccase-mediated crosslinking (Buchert *et al.*, 2010):

4 substrates (reduction) + O_2 ⟶ 4 substrates (oxidation) + 2 H_2O (Eq. 1)

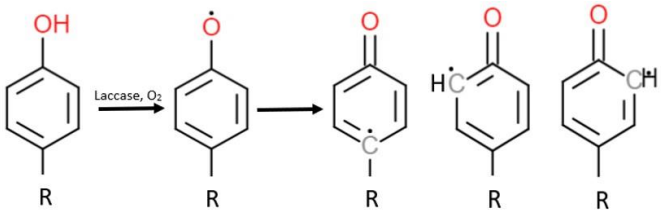

Figure 3. Laccase-mediated crosslinking in food protein.

Laccase-mediated crosslinking has been thoroughly investigated in the past since it can significantly modify food properties. Laccases are seen to significantly improve the stability of emulsions in alkaline environment. The system properties such as colour and solubility are also affected due to the crosslinking between laccase and small reactive phenolic compounds present in globular protein (Isaschar-Ovdat and Fishman, 2018). In addition to its functional properties, laccase is also used for environmental management as it catalyses a wide variety of substrates. This enzyme promotes sustainable development as O_2 is an electron acceptor, while water is the only major product that is formed (Jeon and Chang, 2013). On the other hand, the use of laccase in food matrices is restricted since various metal ions such as Fe^{3+}, Ag^{+}, and Al^{3+} could inhibit its activity, and produce conformational changes. Additionally, some anions such as Cl^{-} and NO^{3-} could also inhibit the catalytic activity of this enzyme, however, the effects are reversible (Li *et al.*, 2020).

Tyrosinase

Tyrosinase, also known as monophenol, *o*-diphenol oxygen oxidoreductase (EC 1.14.18.1), is generally present in animals, plants, microorganisms, and even humans. Bacterial tyrosinases are either monomeric (*Streptomyces* sp.) or dimeric (*Bacillus thuringiensis*) in nature, whereas eukaryotic tyrosinases are multimeric. The monomeric tyrosinases isolated from *Agaricus bisporus* has garnered a lot of attention. These enzymes have a molecular mass of 43 and 13.4 kDa (Claus and Decker, 2006). On the other hand, the molecular weight of the monomeric fungal tyrosinase isolated from *Trichoderma reesei* is 43 kDa (Li *et al.*, 2020). Tyrosinase isolated from mammals is considered the largest tyrosinase with a molecular mass ranging between 62 and 67 kDa (Kim and Uyama, 2005). The optimal pH of microbial and plant tyrosinases is slightly acidic, whereas a low tyrosinase activity is observed at temperatures higher than 70°C.

Tyrosinase is a metalloenzyme containing a Cu atom. It shows a similar activity as the Type-3 Cu protein. It belongs to the copper oxidase enzyme family, and contains six His residues which form a quadruple-helix bundle. It also coordinates two Cu ions (CuA and CuB) at the conserved active site (Sendovski *et al.*, 2011). Tyrosinase is categorised as a bifunctional enzyme that catalyses the *o*-hydroxylation of monophenols (monophenolase or cresolase activity), and also oxidises diphenol molecules (diphenolase activity) to quinones in the presence of molecular oxygen (Figure 4). This molecular oxygen is the electron acceptor in the redox reaction as it gets reduced to H_2O without leading to the production of hydrogen peroxide, *i.e.*, H_2O_2 (Buchert *et al.*, 2010). The redox reaction indicates that one mole of O_2 can oxidise one mole of monophenol, while 0.5 mole of O_2 oxidises one mole of the diphenol compound. Quinone is produced at the end of both reactions. The reactive quinone that is generated due to the diphenolase activity is crosslinked with various groups such as other tyrosine amides, amines, and sulfhydryl (Selinheimo *et al.*, 2008). This reaction leads to the formation of an inter- and intra-molecular bond by the aryloxy free radical coupling, arylalkyl addition, or di-tyrosine formation

(Isaschar-Ovdat and Fishman, 2017). Eq. 2 presents the hydroxylation reaction involving monohydroxy phenols, wherein AH_2 was the reductant:

2 monohydroxy phenols + O_2 + AH_2 ⟶ 2 *o*-dihydroxyphenyls + H_2O + A (Eq. 2)

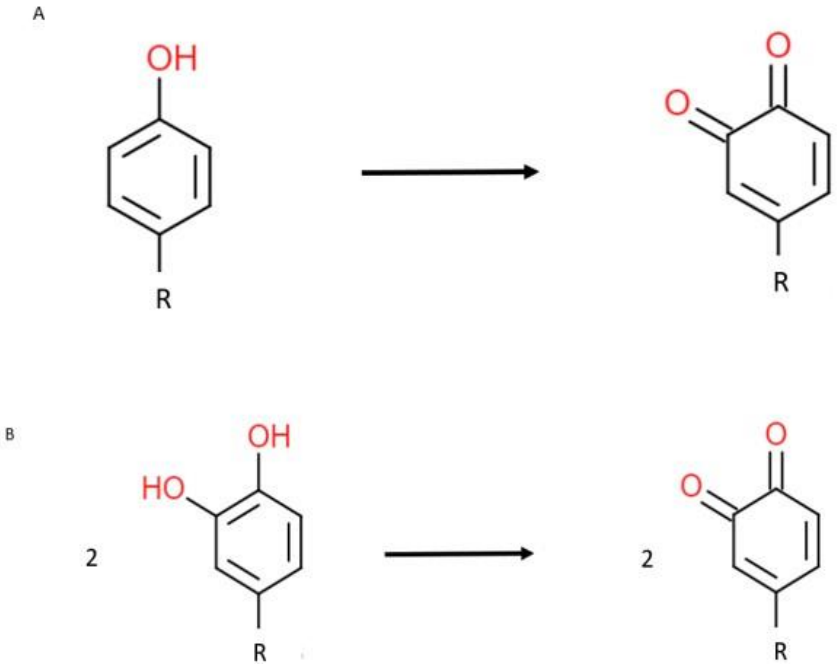

Figure 4. Tyrosinase-mediated crosslinking; **(A)** monophenolase, and **(B)** diphenolase.

Tyrosinase-mediated crosslinking can enhance the properties of food products such as improving the antimicrobial activity, antioxidating activity, higher stability of the nanoparticles or emulsions, resistance to pepsin, better water retention, and increased mechanical strength of the gels (Li *et al.*, 2020). Additionally, this crosslinking does not lead to any environmental pollution. Though the tyrosinases can effectively crosslink with low-complexity food proteins, they cannot catalyse the reactions with proteins having a complex or folded structure. Hence, a small molecular weight compound (*e.g.*, caffeic acid or phenol) is added as a mediator in these reactions (Fairhead and Thöny-Meyer, 2010). The tyrosinase-mediated crosslinking includes *o*-quinones which are not easily controlled. This yields a complex mixture of reaction products, such as monomeric or oligomeric phenols.

Peroxidase

Peroxidases (EC 1.11.1.x) belong to the oxidoreductase group of enzymes which are found in microbial, mammalian, and plant cells (Buchert *et al.*, 2010). 13 major groups of peroxidase family have been discovered, and are further classified into more than 60 classes. Hence, each member of the peroxidase family has a different EC number based on the function, property, and regulation. Peroxidase shows a wide specificity for different electron donors used during the oxidation reaction, however, H_2O_2 is a popular electron acceptor, while the phenolic compounds and amines are used as hydrogen donors. Many peroxidases contain a heme cofactor containing iron protoporphyrin IX in their active site; however, some peroxidase active sites consist of vanadium, selenium, magnesium, and flavin groups (Hofrichter *et al.*, 2010).

Peroxidase catalyses the oxidation of several inorganic and organic substrates which include aromatic compounds such as thiols, phenols, and indoles (Veitch, 2004). This enzyme extracts the single electrons from the substrates to yield free radicals. Simultaneously, H_2O_2 is reduced to generate H_2O molecules (Figure 5). It was noted that one mole of water molecule is generated after the reduction of hydrogen peroxide when two substrate molecules are oxidised by peroxidase. The phenoxy radical that is formed reacts via the coupling reaction which yields a quinone (Hofrichter *et al.*, 2010). This quinone undergoes a non-enzymatic polymerisation with the thiol and amino residues in the food products. This reaction leads to the formation of various bonds such

as the dityrosine and diferulate linkages with the polysaccharide molecules (Zeeb *et al.*, 2012). Eq. 3 describes the reactions that can be catalysed by peroxidase, where AH_2 represents the substrate and $AH^{\cdot}$ represents the free radical products:

$$H_2O_2 + 2\ AH_2 \longrightarrow 2\ H_2O + 2\ AH^{\cdot} \quad \text{(Eq. 3)}$$

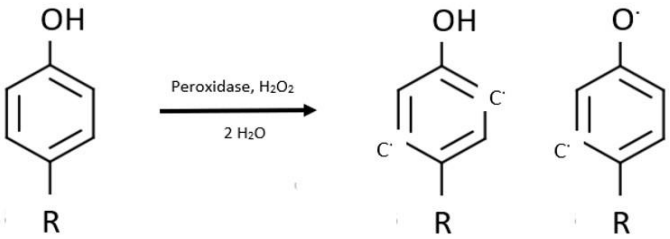

Figure 5. Peroxidase oxidation using H_2O_2 as substrate.

Horseradish peroxidase (HRP) (EC 1.11.1.7) is the most popular enzyme belonging to this family. It is a low redox-potential heme peroxidase which catalyses the oxidation of one electron in the tyrosine residues present in the protein molecules. The tyrosyl radicals that are formed induce the (iso)di-tyrosine conjugation, which leads to the formation of intra-molecular covalent bonds. HRP is favoured in the food industry owing to several main advantages such as the peroxidase-catalysed reaction is dependent on the supply of co-substrate H_2O_2. In this reaction, the H_2O_2 concentration can be easily controlled as compared to the dissolved oxygen concentration. This helps in controlling the crosslinking by altering the addition period and H_2O_2 concentration (Dhayal *et al.*, 2014). Furthermore, HRP-mediated crosslinking enhances the antibacterial and antioxidating activities, thermal sensitivity of the resultant complex, and the stability of the emulsions (Teixeira *et al.*, 2012). The gel formation which is catalysed by HRP displayed effective cytocompatible properties, and was best suited for the colloidal systems. HRP is not used in regular reactions though several researchers have investigated its potential application. This is attributed to the fact that pure HRP extraction on a large scale is not easy nor economical (Jin *et al.*, 2010).

Application of protein crosslinking

Protein crosslinking in meat processing

Cooking does not only enhance the aroma, colour, and palatability of meat, but also makes it microbiologically safer (Lund and Ray, 2017). However, unintentional and undesired outcomes such as loss of essential amino acids and generation of hazardous compounds take place under the influence of thermal processing which causes a detrimental effect on the nutritional quality and safety of meat (Trevisan *et al.*, 2016). In the past few years, value-added meats have garnered a lot of commercial importance in the meat processing industry. The consumer demand for a moderately-priced and high-quality product has led to the investigation of crosslinking methods. These methods help in effectively using the carcass by the value addition of low-value meats. This technique is regarded as restructuring, which includes the gluing together of meat pieces (Buchert *et al.*, 2010). Various crosslinkers are used commercially to improve the texture of meat and meat products.

Traditionally, phosphates and salt, along with heat treatment techniques were used for binding the meat pieces. Unheated comminuted meat products are frozen to enhance their binding. Though the thermal processing technique can affect protein crosslinking, it is not very popular since consumers have become quite health conscious. As a result, food developers are more pressured to develop novel health-oriented products. In their study, Mitra *et al.* (2018) stated that the deamidation and the glycation reactions were more frequent when the food products were heated from 58 to 160°C, which altered the nutritional value of the processed muscle-based foods. Hence, new technologies were designed for eliminating the necessity to carry out heat treatment. Crosslinking mediated by recombinant microbial transglutaminase (MTG) derived from *Pichia pastoris* strain GS115 showed improved hardness, chewiness, and adhesiveness in chicken myofibrillar protein (Yang and Zhang, 2019). MTG can crosslink soy protein isolate component acidic subunits, subunits of β-conglycinin, myofibrillar protein myosin heavy chain, and actin. Canto *et al.* (2014) noted that when TG was added for restricting the caiman steaks, the textural properties and cooking yield could be improved without altering the flavour or colour. Hence, they suggested the combined use of TG and $MgCl_2$ for decreasing the salt concentration without compromising on the sensory attributes (Santhi *et al.*, 2017). Despite using enzymatic crosslinker, Wang *et al.* (2018c) used 2,20-azobis(2-amidinopropane) dihydrochloride (AAPH) for protein crosslinking that subsequently affected the gelling properties of rabbit meat myofibrillar proteins.

Protein crosslinking in fish processing

Fishery resources worldwide are affected by the high demand for fish and fish products which drastically reduce the stocks of many fish species. However, there are several underutilised fish species and filleting by-products that should be transformed into high-value products through surimi technology and restructuring technology. This process includes milling of fish muscle, solubilisation of fish proteins with salt, formatting of fish paste, and induction of the gelling phenomenon using heat treatment (Ramírez *et al.*, 2011).

Enzyme-aided restructuring (Buchert *et al.*, 2010) or used together with hydrocolloids (Ramırez *et al.*, 2002) has become a feasible technology. Ogawa *et al.* (2017) showed D-allulose, which is the C3-epimer of D-fructose, improved the gel strength and water-holding capacity of surimi prepared from marine fish. Prodpran *et al.* (2012) compared the effectiveness of several phenolic compounds in which tannic acid exhibited the highest crosslinking capability on myofibrillar protein. The performance of tannic acid was evidenced by a higher decrease in free amino groups with coincidentally lower band intensity of the myosin heavy chain. Currently, many novel TG-mediated restructured fish products, which contain pieces of various fish species or non-fish options, are widely available in the markets. TG induces an extensive crosslinking of the myosin heavy chain proteins which leads to the formation of gels that display good mechanical properties (Ramírez *et al.*, 2011).

Protein crosslinking in dairy products

Recently, milk protein modification has been a trend in the dairy field. The application of innovative technologies is emerging to produce dairy products with improved technological, rheological, functional, and sensory attributes, for example yield, heat stability, water, fat binding capacity, and gel-forming properties. In particular, protein modification in curd and cheese yields has become a matter of great interest as a considerable number of proteins are lost in whey during rennet clotting (Fox *et al.*, 2017). Loi *et al.* (2018) reported that the activity of purified recombinant laccase Ery4 significantly increased curd weight and antioxidant activity, both before and after gastro-pancreatic digestion. Also, Italian fresh Giuncata cheese treated with laccase and chlorogenic acid showed increased yield (+12%), solid recovery (+21%), dry matter (+18%), and protein content (+13%), as well as decreased hardness and chewiness (Loi *et al.*, 2020). Mozzarella cheese added with carboxymethyl cellulose had a smoother surface but a lower degree of protein crosslinking, hence, glutamine transaminase was added to improve hydrophobic interaction (Li *et al.*, 2019).

Covalent bond formation in a gel network due to enzymatic catalysis improves its firmness, which further enhances the sensory and structural properties of the acid milk gels. During yoghurt production, enzymatic crosslinking is carried out for replacing the addition of non-fat dry matter and stabilisers, or decreasing the dry matter concentration without altering the textural properties of the product. Many researchers have investigated the use of TG with the yoghurt starter culture and chemical acidifier-induced milk gels (Buchert *et al.*, 2010). Yoghurt with stronger acid gel and higher consistency, cohesiveness, index of viscosity, and apparent viscosity was produced through enzyme-mediated crosslink using *Zea mays* TG derived from *Pichia pastoris* GS115 (Li *et al.*, 2017). Abou-Soliman *et al.* (2017) reported that the simultaneous addition of TG at a concentration of 0.4% with a starter culture to fortified camel milk had successfully produced set-type yoghurt. In another research, it has been proven that TG had no effect on *Lactobacillus* sp. in yoghurt, but had a variable effect on *Streptococcus thermophilus*, depending on the duration of enzymatic activity (Ziarno and Zareba, 2020). Wang *et al.* (2018b) revealed that an increase of crosslink of kappa-casein and alpha-lactalbumin with TG treatment was beneficial for yoghurt stability in terms of pH, viscosity, and water holding capacity. The effect was reported to be more obvious with increasing TG concentration.

Protein crosslinking in bread

Bread made from wheat flour is one of the most consumed foods worldwide, especially in Western countries (Carcea *et al.*, 2018). Nevertheless, there is a trend towards gluten-free bread due to growing gluten intolerance, gluten allergy, and celiac disease. In recent years, gluten-free bread has become the topic of interest owing to the high amounts of starch without the presence of gluten. However, the available proteins do not have the viscoelastic and structuring properties of gluten (Martins *et al.*, 2019). An alternative to improve the properties of bread could be the use of crosslinkers to enhance protein crosslinking in the product.

Gusmão *et al.* (2019) reported that bread produced using red rice flour and cassava flour, TG, and chitosan at concentrations of 0, 1, and 2%, respectively, had lighter brown colouration due to incomplete Maillard reaction as well as low specific volumes. In addition, TG produced isopeptide bonds especially in the gluten fraction, thus resulting in the formation of protein aggregates which improved the structure of bread (Ogilvie *et al.*, 2021). TG combined with sourdough exhibited a positive synergistic effect, thus allowing the production of flavour-enriched bread with a longer shelf-life (Scarnato *et al.*, 2017). TG improved dynamic moduli and gelatinisation temperatures of bread made of hemp protein as well as enhancing onset gelatinisation when soy protein was used (Baldino *et al.*, 2020). Nutritional benefit and technological quality of millet (*Panicum miliaceum*) bread such as structure strengthening, specific volume, and sensory quality were also improved when TG ranging from 0.5 to 1.5% was added to millet flour (Tomić *et al.*, 2020). Manhivi *et al.* (2018) used laccase to decrease thiol and total phenolic contents by up to 28 and 93%, respectively, as elastic and viscous modulus of dough increased significantly due to laccase-mediated crosslinking of proteins. The effect of laccase from *Trametes maxima* CU1 on the physicochemical quality of bread was also evaluated and successfully used to improve height, weight loss, and hardness of bread (Niño-Medina *et al.*, 2017).

Protein crosslinking in pastas and noodles

Noodles are made by dough sheeting process using common wheat (*Triticum aestivum* L.) flour; pasta is made from durum wheat (*Triticum turgidum* L. var. *durum*), and semolina is made from dough extrusion (Fu, 2008). The high production and consumption of food products such as noodles and pastas are attributed to its ease in transportation, production mechanisation, preparation, cooking, or infrastructure development. Due to the diversity of these food products, food developers use pre-treatments or reinforcing compounds for improving the dough rheological and physicochemical properties (Gulia *et al.*, 2014).

TG has gained much attention as an enzymatic crosslinker in pasta and noodle production owing to the advantages earlier mentioned. TG in the concentration of 3 and 4% activated protein crosslinking and increased cooking yield, hardness, and tensile strength, while decreasing cooking loss and adhesiveness of alkaline rice noodle (Gharibzahedi *et al.*, 2019). Upon increasing TG concentration in wheat noodles, a denser protein network with increased connectivity, supported by a decrease in protein solubility and gelatinisation enthalpy, and increased firmness and work of shear were observed (Wee and Jeyakumar Henry, 2019). Kumar *et al.* (2019) reported that the combination of sodium caseinate, whey protein concentrate, and TG decreased the cooking loss, water absorption, and pasting properties, but increased L*, a*, b* and sensory attributes of gluten-free pasta.

Natural crosslinking was also reported in noodles as disulphide bonds were observed in egg yolk and egg white noodles, thus resulting in protein crosslinking and improved functionality (Lambrecht *et al.*, 2017). This is supported by Guo *et al.* (2020) as the addition of egg white significantly improved hardness, chewiness, tensile force, and tensile distance of oat noodles. In addition, Guo *et al.* (2017) showed the occurrence of physical crosslinking in which the addition of alkali improved the dynamic rheological properties in terms of elastic and viscous modulus as well as producing a tight and continuous protein network in buckwheat noodles.

Others

Potato is rich in lysine, tyrosine, and polyphenols; hence, it has good theoretical feasibility to induce protein crosslinking of potato by using an enzyme such as TG, laccase, tyrosinase, and peroxidase (Vaz Patto *et al.*, 2015). TG- and peroxidase-mediated protein gels had more homogeneous three-dimensional networks with larger pore sizes, meanwhile, laccase- and tyrosinase-treated protein gels displayed less homogenous networks with smaller pore sizes. Similarly, TG and peroxidase gave higher storage modulus and increased gel strength as compared to laccase and tyrosinase (Gui *et al.*, 2020). Glusac *et al.* (2017) reported that tyrosinase-mediated potato protein crosslinking on the properties of emulsions had improved droplet size distribution, rheological behaviour, creaming resistance, and microstructure. Finally, tyrosinase derived from *Bacillus megaterium* enhanced the texture of soy glycinin by modulating the protein gel properties through crosslinking (Isaschar-Ovdat *et al.*, 2016).

Physiological impact of protein crosslinking

Very few researchers have investigated the digestibility, allergenicity, and similar physiological effects of protein crosslinking. The determination of amino acid sequences which affect the antibody binding can differ owing to the development of new intra- or inter-molecular bonds, which leads to the formation of novel antigenic sequences or elimination of the allergens. Thus, the digestion rate decreases in the presence of novel covalent bonds, which improves energy intake and satiety levels. Levi *et al.* (2017) reported significant alteration in *in vitro* digestion of protein. This was attributed to the conformation changes that occurred upon adsorption to droplet interfaces. In addition, Fang *et al.* (2020) suggested that enzymatic crosslink results in firmer matrices that are digested to a lower extent.

The addition of TG led to decreased digestibility of chickpea protein due to the presence of isopeptide bonds and the chemical nature of the crosslinks (Monogioudi *et al.*, 2011; Glusac *et al.*, 2020). The tight and compact structure of proteins formed via TG-mediated crosslinking may explain the reduced digestion. Furthermore, reduced digestibility was found after TG crosslinked other legume proteins (Romano *et al.*, 2016). However, crosslinked chickpea emulsion was digested at the end of the gastric phase due to the breakdown of crosslinks, and additional enzyme cutting sites being exposed to pepsin, thus resulting in enzymatic hydrolysis (Fang *et al.*, 2020). Tang *et al.* (2006) reported that protein crosslinking primarily affected *in vitro* pepsin digestion pattern of various subunits of β-conglycinin, whereas the trypsin digestion pattern of native soy protein isolate remains almost unaffected.

Scientists find it challenging to seek modifications that would decrease the immunogenic response and decrease indigestion caused due to common allergens such as milk proteins (Bu *et al.*, 2013). In their report, Monogioudi *et al.* (2011) studied the effects of TG and the tyrosinase crosslinking on β-casein digestibility, wherein the crosslinked β-casein showed higher resistance to pepsin, and was very stable under acidic conditions as compared to non-crosslinked protein. Additionally, Stanic *et al.* (2010) addressed the impact of enzymatic crosslinking on IgE binding and allergenicity of β-casein. The crosslinking was mediated by tyrosinase, TG, tyrosinase-caffeic acid combination (TyrAb-CA), and laccase derived from *Trametes hirsute*, and combined with caffeic acid (ThL-CA). They observed the formation of high molecular weight polymeric compounds, wherein tyrosinase and TyrAb-CA displayed a substantial crosslinking reaction and development of large polymers. The highly polymerised caseins showed a high potential for IgE binding inhibition, which decreased its allergenicity potential.

Ahmed *et al.* (2020) studied the effect of crosslinking shrimp on its potential allergenicity. They noted the formation of macromolecules by crosslinked tropomyosin with TG and laccase that altered the allergen's conformation. ELISA and Western blots showed a decrease in the IgG/IgE binding potential of the crosslinked tropomyosin. Enzymatic crosslinking improved gastrointestinal digestibility, and reduced the degranulation level in RBL-2H3 and KU812 cells. Also, crosslinking of shrimp tropomyosin by TG and tyrosinase reduced the IgE-induction capacity of tropomyosin but maintained the IgG-induction ability, thus making the treated tropomyosin potentially hypoallergenic (Wang *et al.*, 2019).

Radosavljevic *et al.* (2014) revealed that aggregation of proteins via tyrosinase-mediated crosslinking did not change the immunological properties of peanut extract and induction of food allergic responses. The aggregation of peanut allergens did not reduce the specific binding of IgE antibody and transepithelial transport of allergens *in vitro*, did not promote an allergic response *in vivo*, nor compromised the capacity of peanut proteins to induce low dose oral tolerance. Thus, the results showed that covalent crosslinking of proteins otherwise prone to aggregate may be a safe approach in food processing.

Conclusion

Recently, increasing demand to produce nutritious foods with pleasurable textures and mouthfeel has become a challenge in the food industry. Researchers have since expressed more interest in investigating the optimal food texture while developing healthy food products, and having low sugar and fat contents. Their research indicated that a protein modification technique focused on enzymatic crosslinking could help in changing the fat or protein concentration in the food products while maintaining the sensory and physical attributes.

Enzyme technology offers a higher potential for altering the basic properties of food constituents such as polysaccharides, proteins, and phenolics, thus leading to improved functional performance. In the present review, the applications of various enzymes such as laccases, transglutaminases, tyrosinases, and peroxidases for improving the functional properties of food products such as fish, meat, cereals, and dairy-based products were discussed. Each of these enzymes has its unique catalytic mechanism, optimal operating conditions, substrate range, and potential applications. For instance, TG needs the presence of Lys and Gln residues, while laccases and peroxidases require the addition of small molecular weight mediators which help in crosslinking the globular proteins. Tyrosinases derived from one source exhibit higher crosslinking and improve the protein texture, whereas tyrosinases derived from different sources exhibit lower activity on that particular substrate.

Currently, very few researchers have investigated the optimal reaction conditions for enzymatic crosslinking of specific food molecules. Additional experiments such as cell culture, *in vitro* digestion, and animal feeding experiments, therefore, need to be conducted. These would help in determining the safety and potential efficacy of the protein crosslinking reaction. Also, the recycling and the cost of the enzymes could limit the development of enzymatic protein modification techniques. The enzyme immobilisation technique could help in resolving this issue, however, further research needs to be conducted on this topic.

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