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Creating Proteins with Novel Functionality via the Maillard Reaction: A Review

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Proteins are widely utilized to add functional properties, such as gelling and emulsification to foods. These attributes depend on a number of factors such as molecular structure of the protein, the pH, and the composition of its chemical environment. There is substantial evidence to suggest that the functional properties of food proteins can be further improved by derivatization. Covalent bonding of proteins to polysaccharides and smaller reducing sugars via the Maillard reaction has been shown to alter the functionality of proteins without requiring the addition of chemical reagents. Establishment of a technologically feasible method for preparing the conjugates and optimization of the processing conditions, however, is needed to promote their development as functional food ingredients. This paper provides a state-of-the-art contribution to the impact of the Maillard reaction on protein functionality. It presents a deeper understanding of the influence of processing conditions and reactant formulation on improving desirable properties of proteins. In particular attention is given to how potential improvements could be achieved in the emulsifying, textural, and solubility properties of proteins to add value to commodity food ingredients. Elements that are considered to be critical to the design of functional Maillard conjugates are highlighted and suggestions proposed to facilitate progress in this area.

Keywords glycation, functional properties, cross-linking, food, glycoconjugates, protein modification

INTRODUCTION

Proteins are widely utilized in the food industry due to their ability to add unique functional properties such as emulsifying, foaming, gelling and solubility attributes (Phillips et al., 1994). These diverse properties depend on both intrinsic (e.g. molecular structure, composition) and extrinsic (e.g. temperature, chemical environment, pH) factors (Damodaran, 1996). It has been well established that the functional properties of proteins can be further improved by covalent bonding with polysaccharides and smaller sugars (Akhtar and Dickinson, 2003; Aoki et al., 1999; Campbell et al., 2003; Dickinson and Izgi, 1996; Gerrard et al., 2003b; Kato et al., 1995; Manzocco et al., 2000; Shepherd et al., 2000; Sun et al., 2004). Conjugation is based on the Amadori rearrangement steps in the Maillard reaction. The Maillard reaction was first observed by the French chemist, Louis-Camille

Maillard (Maillard, 1912), and involves a complex network of non-enzymatic reactions resulting from the initial condensation between an available amino group and a carbonyl-containing moiety, usually a reducing sugar.

The Maillard reaction is a spontaneous and naturally occurring reaction, in contrast to acetylation, deamidation, succinylation, and other chemical methods available to improve the functional properties of proteins. It is greatly accelerated by heat and no extraneous chemicals are required. Hence the Maillard reaction is probably the most promising approach to improve the functional properties of proteins for food purposes. However, industrially-feasible methods of preparing the Maillard glycoconjugates are needed in order to exploit the potential of the Maillard reaction for the creation of value-added food ingredients.

The aim of this review is to provide a state-of-the-art contribution to the potential impact of the Maillard reaction on protein functionality. Its purpose is to present a deeper understanding of the influence of processing conditions and reactant formulation on the emulsifying, textural, and solubility properties of proteins.

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ABBREVIATIONS

AMP	(advanced Maillard products)
a_w	(water activity)
BSA	(bovine serum albumin)
CMC	(carboxymethyl cellulose)
1-DH	(1-deoxy-2,3-diketose)
3-DH	(3-deoxyaldoketose)
4-DH	(4-deoxy-2,3-diketose)
DOGDIC	(N^6 -{2-[(4S)-4-ammonio-5-oxido-5-oxopentyl]-amino}-5-[(2S,3R)-2,3,4-trihydroxybutyl]-3,5-dihydro-4H-imidazol-4-ylidene}-L-lysinate)
DX	(dextran)
EA	(emulsifying activity)
EP	(emulsifying properties)
ES	(emulsifying stability)
EW	(egg white)
GlcUA	(glucuronic acid)
Glucosepane	(2-ammonio-6-{2[(4-ammonio-5-oxido-5-oxopentyl)amino]-6,7-dihydroxy-4,5,6,7,8,8a-hexahydroimidazo[4,5-b]azepin-4-yl}hexanoate)
GM	(galactomannan)
GODIC	(2-ammonio-6-{2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-4,5-dihydro-1H-imidazol-5-ylidene}amino)hexanoate)
GOLD	(2-ammonio-6-[1-(5-ammonio-6-oxido-6-oxohexyl)imidazolium-3-yl]hexanoate)
HMW	(high molecular weight)
α -LA	(α -lactalbumin)
B-LG	(β -lactoglobulin)
LMW	(low molecular weight)
MODIC	(2-ammonio-6-{2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-4-methyl-4,5-dihydro-1H-imidazol-5-ylidene}amino)hexanoate)
MOLD	(2-ammonio-6-[1-(5-ammonio-6-oxido-6-oxohexyl)-4-methylimidazolium-3-yl]hexanoate)
MW	(molecular weight)
OVA	(ovalbumin)
o/w	(oil-in-water)
Pentosidine	(2-ammonio-6-{2-[(4-ammonio-5-oxido-5-oxopentyl)-amino]-3H-imidazo[4,5-b]pyridin-4-ium-4-yl}hexanoate)
RH	(relative humidity)
WPI	(whey protein isolate)
XG	(xyloglucan)

PROTEIN GLYCATION

Glycation resulting in the attachment of sugar residues to proteins via the Maillard reaction can affect the functional properties of proteins by causing a change in their charge, solvation, and/or conformation (Darewicz and Dziuba, 2001; Kato

et al., 1993; Khan et al., 1999; Morgan et al., 1999; Nakamura et al., 1994). Additionally it can also result in cross-linking of protein molecules (Chellan and Nagaraj, 1999; Faye et al., 2000; Friedman, 1996; Gerrard and Brown, 2002; Gerrard et al., 2003a and b; Lederer and Bühler, 1999; Meade et al., 2003). In most foods it is the ϵ -amino group of the lysine residues in proteins that are the primary source of reactive amino groups. The imidazole group of histidine, the indole group of tryptophan, and the guanidino group of arginine residues also react but to a lesser extent (Ames, 1992).

The glycoconjugates formed via the Maillard reaction are not a single species. They consist of various glycoforms and are produced in combination with a vast array of predominantly poorly-characterized products. The types and yields of Maillard reaction products are further influenced by various reaction conditions, including temperature, pH, amino:carbonyl ratio, relative humidity (RH) and the intrinsic properties of the reactants (Labuza and Baisier, 1992; Van Boekel, 2001).

Studies focused on the functionality of Maillard glycoconjugates have been numerous and include both beneficial and detrimental outcomes. For example, protein glycation during the early stages of the Maillard reaction, has been shown to increase emulsifying activity (EA) (Darewicz and Dziuba, 2001; Diftis and Kiosseoglou, 2004; Dickinson and Galazka, 1991; Groubet et al., 1999; Kato et al., 1992), improve foaming properties (Dickinson and Izgi, 1996), increase calcium complexing activity (Aoki et al., 1994), increase protein solubility (Katayama et al., 2002; Saeki and Inoue, 1997; Sato et al., 2000; Shepherd et al., 2000), enhance antimicrobial/bactericidal activity (Nakamura et al., 1998; Song et al., 2002; Takahashi et al., 2000), and increase heat stability (Aoki et al., 1999; Chevalier et al., 2001b; Hattori et al., 1997). Beneficial effects have also been associated with advanced stages of the Maillard reaction, including the formation of compounds with antioxidant (Chevalier et al., 2001a; McGookin and Augustin, 1991; Nakamura et al., 1998; Okamoto et al., 1992), anticarcinogenic, and antimutagenic properties (Usman and Hosono, 1998). The cross-linking of proteins has been associated with improved food texture (Ashie and Lanier, 1999; Gerrard et al., 2003b).

On the other hand, it has been reported that glycation can result in formation of mutagenic compounds (Brands et al., 2000). Hence any food ingredient created by the Maillard reaction requires thorough safety testing to rule out the presence of such compounds. It decreases nutritional value due to blocking of lysine and other essential amino acids (Malec et al., 2002; Pizzoferrato et al., 1998). It may cause off-flavor development (Morr and Ha, 1991; Walker, 1972), poor protein solubility (Kato et al., 1986; Lea and Hannan, 1949 and 1950), and decreased digestibility (Culver and Swaisgood, 1989), produce adverse browning (Guerra-Hernandez et al., 2002; Saio et al., 1980), and shorten product shelf-life (Albalá-Hurtado et al., 1999; Guyomarc'h et al., 2000; Stapelfeldt et al., 1997; Van Mill and Jans, 1991).

Irrespective of the consequences, these studies illustrate the potential for using glycation to manipulate protein properties.

However, they leave unanswered interesting questions regarding conditions required for industrially feasible processes to improve the functional properties of proteins. Obtaining a better understanding of how key reaction parameters affect glycation and the cross-linking process and moreover, their influence on protein functionality, will assist in the development of industrial processes to make novel and value-added food ingredients.

OVERVIEW OF THE CHEMISTRY OF THE MAILLARD REACTION

The first coherent scheme of the Maillard reaction was presented by Hodge (1953). Although still useful, a substantial amount of research has since been undertaken (for example Ames, 1992; Friedman, 1996; Glomb and Tschirnich, 2001; Huyghues-Despointes and Yaylayan, 1996; Labuza, 1994; Ledl, 1990; Ledl and Schleicher, 1990; Namiki, 1988; O'Brien, 1997; Reihl et al., 2004; Tressl et al., 1995; Van Boekel and Brands, 1998; Weenen, 1998; Yaylayan, 1997) culminating in a revised version (Tressl et al., 1995) (Figure 1).

In essence, the Maillard reaction can be summarized into three stages; early, intermediate, and final. The early stage of the Maillard reaction, which is the initial glycation reaction, is well characterized. It involves a condensation reaction between the carbonyl group of a reducing sugar with an available, unprotonated amine group, forming a Schiff base with the release of water (Ames, 1992). The Schiff base that is formed subsequently cyclizes to the corresponding *N*-glycosylamine, which then undergoes an irreversible Amadori rearrangement to produce the Amadori product (1-amino-1-deoxy-2-ketose) (Ames, 1992) (Figure 2). With ketoses, such as fructose, the Heyn's rearrange-

ment changes a ketosylamine to a corresponding Heyn's product (2-amino-2-deoxyaldose) (Martins et al., 2001) (Figure 3). No color is produced at this stage of the reaction.

The intermediate stage begins with the degradation of the Amadori/Heyn's product(s) which can undergo numerous transformations involving various divergent pathways. These include oxidation, fragmentation, enolization, dehydration, acid hydrolysis, and free radical reactions resulting in a large multiplicity of poorly characterized compounds (Friedman, 1996; Ledl and Schleicher, 1990). Although some color is produced at this stage, most of the color is not produced until the final stage when highly colored, water-insoluble, nitrogen-containing polymeric compounds referred to as "melanoidins" are produced (Friedman, 1996).

In contrast to the earlier stages the high degree of complexity of the advanced stages of the Maillard reaction has made their accurate chemical description a formidable quandary. So far the details of this stage are unresolved although the results, in terms of cooking reactions that give color and flavor changes, are easily recognized.

Ideally, a glycoconjugate destined for incorporation into food will possess improved protein functionality with minimal color or flavor development. This requires that the Maillard reaction be performed under carefully controlled conditions to prevent the later stage color and flavor changes.

REACTION CONDITIONS

The Maillard reaction takes place in both solution ("wet") and "dry" conditions. However, optimum water activity (a_w) for

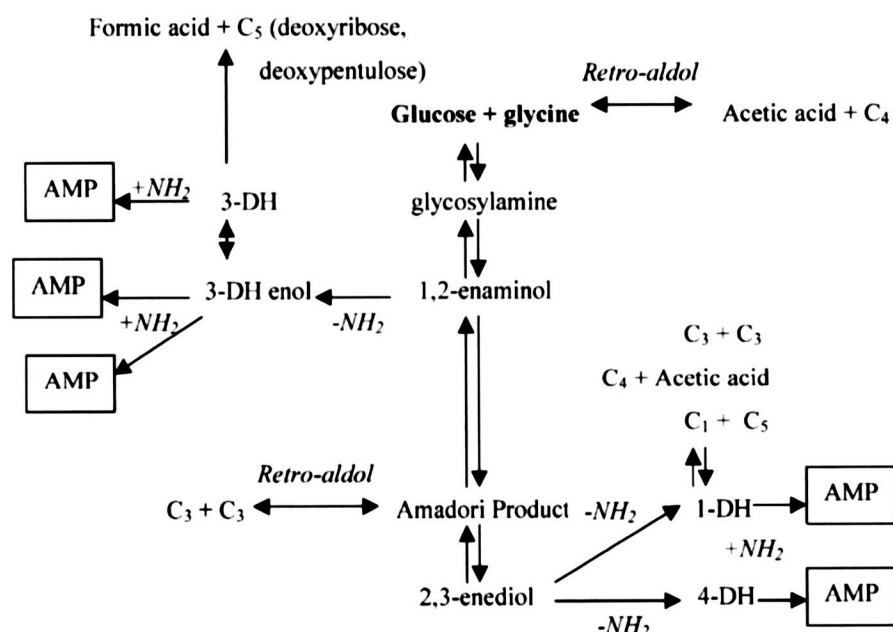


Figure 1 Scheme for glucose-glycine Maillard reaction adapted from Tressl et al. (1995). AMP (Advanced Maillard Products); 1-DH (1-deoxy-2,3-diketose); 3-DH (3-deoxyaldoketose); 4-DH (4-deoxy-2,3-diketose).

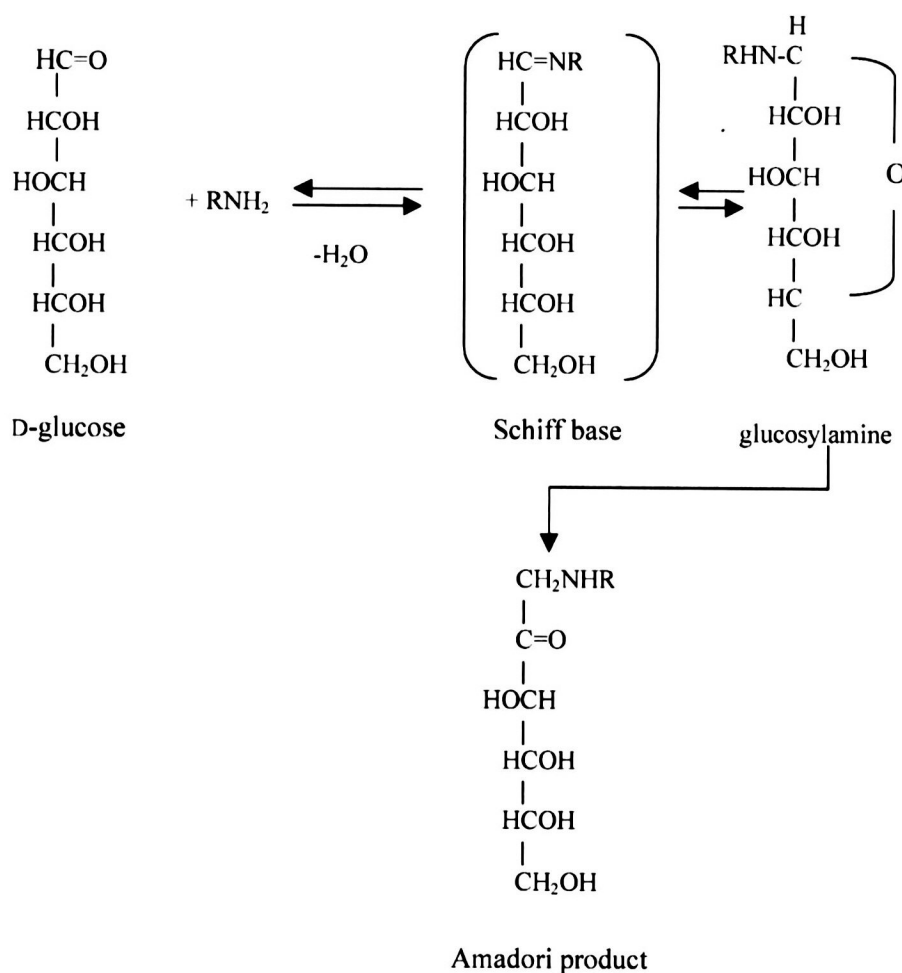


Figure 2 The initial Maillard reaction with glucose (the Amadori rearrangement) (adapted from Dills, 1993).

the Maillard reaction is reported to be between 0.5–0.8 (Labuza and Baisier, 1992; Van Boekel, 2001). At the lower range of this level of a_w protein preparations, without substantial sugar or polyol content, will exist as finely divided powders or loosely agglomerated solids without free water i.e. in a “dry” state.

“Dry” reactions can be carried out by lyophilizing a solution of protein and reducing sugar, followed by heating under controlled temperature and RH conditions for a given time. “Dry” reactions are more desirable from an industrial perspective, based on the premise that they require less space and time to achieve the desired result than “wet” reaction conditions. Moreover, “dry” products provide longer-term stability and greater ease of handling and storage than liquid products. Some “dry” Maillard reactions, however, particularly those involving formation of protein-polysaccharide conjugates derived from globular proteins, are performed over 2–3 weeks (Babiker et al., 1998; Kato and Kobayashi, 1991; Nakamura and Kato, 2000; Nakamura et al., 1994; Song et al., 2002). These extended reactions are not industrially practical. Additionally, laboratory processes usually use freeze-drying for lyophilization followed by equilibration to desired a_w in a controlled RH atmosphere. This may not be practical on an industrial scale and improved technologies are

required. This may involve further optimization of the reaction conditions, and preferably utilize spray-drying or roller-drying for example, to replace the freeze-drying process and produce powders of the required a_w to promote the reaction.

PROTEIN CROSS-LINKING AND THE MAILLARD REACTION

It has long been known that cross-linking of protein chains occurs during the advanced and final stages of the Maillard reaction (Fayle et al., 2000; Gerrard et al., 2003a and b; Hodge, 1953; Lederer and Bühler, 1999). It is only recently, however, that some of these cross-links have been characterized. These include pentosidine, glucosepane, GOLD (glyoxal lysine dimer), MOLD (methylglyoxal lysine dimer), DOGDIC (a lysine arginine dimer), MODIC (methylglyoxal lysine arginine dimer), and GODIC (glyoxal lysine arginine dimer) (Figure 4) (Biemel et al., 2001; Chellan and Nagaraj, 1999; Frye et al., 1998; Nagaraj et al., 1996; Sell and Monnier, 1989; Wells-Knecht et al., 1995). Several investigators have indicated that the sugar-derived dicarbonyl compounds, such as methylglyoxal,

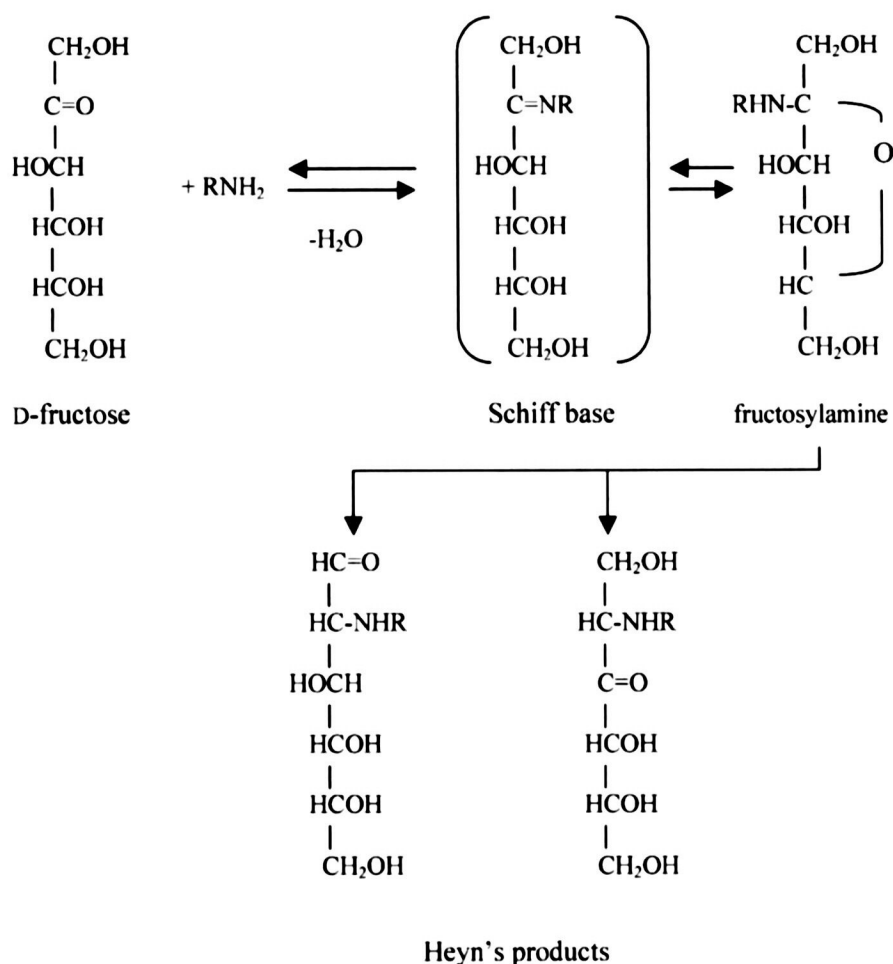


Figure 3 The initial Maillard reaction with fructose (the Heyn's rearrangement). The major Heyn's product is shown to the left (adapted from Dills, 1993).

3-deoxyosones or glyoxal (Biemel et al., 2002; Chellan and Nagaraj, 1999; Grandhee and Monnier, 1991; Lederer et al., 1998; Meade et al., 2003) attach to lysine, arginine, and tryptophan residues of the protein via one of their bifunctional groups (Glomb and Tschirnich, 2001; Hirsch et al., 1995; Hollnagel and Kroh, 2000; Wells-Knecht et al., 1995). The proteins, with the bifunctional agents attached, are then thought to polymerize by binding of the second functional group with the remaining lysine and arginine residues of the protein. The tryptophan residues seem to react only with the first functional group of the bifunctional reagents, i.e. they do not appear to play a role in polymerization of the protein-reducing sugar complexes (Cho et al., 1984; Hollnagel and Kroh, 2002; Kato et al., 1987; Okitani et al., 1984; Shin et al., 1988). Despite extensive research into the molecular mechanism of the protein cross-linking no relationship between the structure of the dicarbonyl moieties and their rate of activity as cross-linking agents has been established (Meade et al., 2003; Miller et al., 2003).

The reactive carbonyl groups of the dicarbonyl moieties that have been implicated in the cross-linking of proteins are positioned side by side in the α -position (Figure 5). For proteins composed of long chains of amino acids, and particularly where

these amino acids have bulky side-chains, it may not always be sterically feasible for a single dicarbonyl species to cross-link two different protein chains at such close proximity along its chain. This would be an even greater challenge for proteins having low flexibility. It may be postulated, therefore, that the dicarbonyls involved in Strecker degradation reactions may only be partially responsible for cross-linking of protein via the Maillard reaction.

CHANGES IN TEXTURAL AND GELATION PROPERTIES

It has been shown that heating solutions of globular proteins (e.g. lysozyme, bovine serum albumin (BSA), soy) in the presence of low molecular weight reducing sugars, such as lactose, ribose, or xylose causes the formation of gels that show higher breaking strengths than comparable samples heated in the absence of reducing sugars (Armstrong et al., 1994; Cabodevila et al., 1994; Easa et al., 1996a and 1996b; Hill and Easa, 1998; Mitchell and Hill, 1995; Rich and Foegeding, 2000). The more

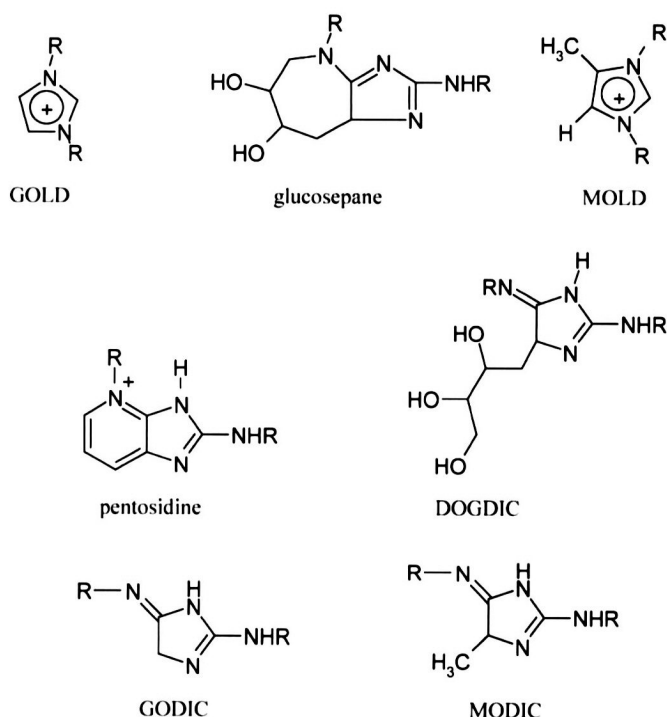


Figure 4 Proposed heterocyclic protein cross-link structures (adapted from Meade et al., 2003). GOLD, 2-ammonio-6-[1-(5-ammonio-6-oxido-6-oxohexyl)imidazolium-3-yl]hexanoate; glucosamine, 2-ammonio-6-[2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-6,7-dihydroxy-4,5,6,7,8,8a-hexahydroimidazo[4,5-b]azepin-4-yl]hexanoate; MOLD, 2-ammonio-6-[1-(5-ammonio-6-oxido-6-oxohexyl)-4-methylimidazolium-3-yl]hexanoate; pentosidine, 2-ammonio-6-[2-[(4-ammonio-5-oxido-5-oxopentyl)-amino]-3H-imidazo[4,5-b]pyridin-4-ium-4-yl]hexanoate; DOGDIC, N^6 -{2-[(4S)-4-ammonio-5-oxido-5-oxopentyl]amino}-5-[(2S,3R)-2,3,4-trihydroxybutyl]-3,5-dihydro-4H-imidazol-4-ylidene-L-lysine; GODIC, 2-ammonio-6-[(2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-4,5-dihydro-1H-imidazol-5-ylidene)amino]hexanoate; MODIC, 2-ammonio-6-[(2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-4-methyl-4,5-dihydro-1H-imidazol-5-ylidene)amino]hexanoate.

reactive the reducing sugar, as classified by the Maillard reaction (Ames, 1992; Labuza and Baisier, 1992; Martins et al., 2001; O'Brien, 1997), the higher the gel strength but the darker the colour (Hill et al., 1992).

The increase in gel strength can be attributed to the sugar-induced covalent cross-linking of proteins. Hence, in the presence of reducing sugars gelation occurs at low protein concentrations. For example, the minimum amount of BSA required

to form a gel was shown to be 7% but this could be decreased to 1.2% or 0.5% if 3% xylose or 3% ribose respectively, were incorporated into the system (Easa, 1996). Furthermore, by increasing the initial sugar concentration the time for gelation can be decreased. For example it took 160 min to gel a 3% BSA solution in the presence of 2% xylose, whereas with 4% xylose gelation was achieved in 90 min (Easa, 1996).

Recent studies have focused on improving the gelation properties of globular protein powders made by reaction with reducing sugars in the "dry" state. The powders are subsequently dissolved, heated, and aged to form the gel. On heating egg white (EW) with galactomannan (GM) in the "dry" state (3 days at 60°C, 65% RH, 1:4 EW:GM ratio), Matsudomi et al. (2002) showed improved gelation after heating a solution of the conjugate (10% w/v protein) at 90°C for 30 min.

Similarly, whey protein-pectin conjugates which had been heated in the "dry" state for 15 days (60°C, 35–40% RH), formed gels after further heating an aqueous solution of the conjugate (12% w/v protein) at 90°C (10 min) followed by cooling (6–8°C, 24 h) (Mishra et al., 2001). Reaction of de-sugared dried EW with the highly reactive reducing sugar, glucose (55°C, 35% RH, 0–12 day) (Handa and Kuroda, 1999), and ovalbumin (OVA)-psicose, -glucose and -fructose conjugates (55°C, 65% RH, 4 days, 1:20 protein:sugar mole ratio) (Sun et al., 2004) required similar treatments; gels were prepared after heating solutions of the conjugates (8% w/v protein) at 80°C (30–40 min) (Handa and Kuroda, 1999) and subsequent cooling (15 min) (Sun et al., 2004).

Because globular proteins gel only following denaturation, the sugar may protect the protein from denaturation during "dry" heating. On further heating of the glycosylated protein in solution, however, another mechanism appears to be operating which facilitates the cross-linking process. This suggests a spontaneous hydrolytic reaction, such as deamidation, may be involved (Clarke et al., 1992). It is well known that heating proteins and reducing sugars in solution accelerates protein denaturation leading to gross structural modifications of the protein. In contrast, glycation in the "dry" state can be carried out without significantly altering the native-like behavior of the protein (Gauthier et al., 2001; Morgan et al., 1999).

Extensive glycation has been shown to decrease the solubility of proteins due to polymerization and further cross-linking (Aoki et al., 2001; Katayama et al., 2002; Sato et al., 2000; Tanabe and

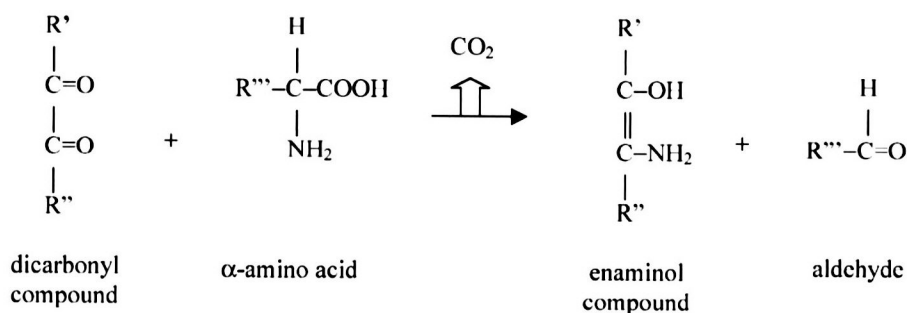


Figure 5 Strecker degradation (adapted from Kurata and Otsuka, 1998).

Saeki, 2001). Thus it seems probable that considerable further improvement to the gelation properties of the proteins could be achieved by carefully controlling the extent of polymerization and cross-linking associated with the advanced Maillard reaction. In this way, glycoconjugates showing an increasing gradation in viscosity from highly liquid to viscous-like to gelled network could be obtained on reconstitution of the samples. Surprisingly, this is an area of research that has received little attention.

One group of proteins that may benefit from this glycation approach are the caseinates. Caseinates do not require denaturation to expose their reactive amino groups and can therefore be expected to react more readily in the Maillard reaction, than globular proteins. Moreover, caseinates are very heat stable (able to withstand 140°C, 20 min) and are required in relatively high concentrations (>10% w/v) to produce viscous solutions (Ennis and Mulvihill, 2000). Hence, endowing caseinates with gelling properties would thus be a highly novel and exciting area of development. Although Colas et al. (1988) and Courthaudon et al. (1989) showed that the viscosity of caseinate could, in some cases, be increased by reductive alkylation in the presence of reducing sugars, a relatively high protein concentration of the conjugate was required (8–10% w/v) and there was a large dependence on the type of reducing sugar. For example, although glycation with glucose was effective in increasing the viscosity of caseinate, the caseinate-fructose conjugate had lower viscosity than caseinate alone. Moreover, the method used by these authors utilizes a solution of highly toxic chemical agents making the process unsuitable for food application.

Preliminary studies have been carried out by Stefancich and Delben (1997) with purified β -casein and the polysaccharide, glutamate glucan. After heating at 60°C and 65% RH for 3 days the viscosity of the β -casein-glutamate glucan conjugate was improved compared to heated glutamate glucan alone. In fact the conjugate gave a flow curve having a consistency factor 2.6-fold higher than that of the polysaccharide. More fundamental scientific studies on the combined effects of caseinates and the Maillard reaction on improving the viscosity of the protein upon "dry" heating, however, could not be found.

EMULSIFYING PROPERTIES

Kato and co-workers (1988, 1990, 1991, 1992, and 1993) were among the first to describe the emulsifying properties (EP) of protein-reducing sugar conjugates. Lysozyme with poor EP in the native form showed highly effective EP after conjugation with dextran (DX) (Kato and Kobayashi, 1991) and GM (Nakamura et al., 1992). The EP of the conjugates were superior to those of commercial emulsifiers, even under acidic or high salt conditions. Furthermore, they were not affected by pre-heat treatment. In fact, in some cases, heating solutions of the conjugates prior to emulsification has shown to further increase their excellent EP (Kato and Kobayashi, 1991; Kato et al., 1990). Since high salt conditions, acidic pH, and

heat treatment are commonly encountered in industrial applications, the lysozyme-polysaccharide conjugates could be considered effective, safe emulsifiers for food processing. Similar reports have been described for BSA-GM (Kim et al., 2003), soy protein-GM (Babiker et al., 1998), caseinate-maltodextrin (Shepherd et al., 2000), OVA-DX (Kato et al., 1990), OVA-GM (Nakamura and Kato, 2000) and protamine-GM (Matsudomi et al., 1994), for example. Whether these functional attributes can be reproduced on a pilot scale was not addressed in these papers. Moreover, presumably due to the limitations in the methodology used to prepare the conjugates as addressed earlier in this review, no such conjugates have yet been commercialised.

In contrast to their reaction with polysaccharides, following glycation with glucose α_s -casein (10:1 w/w, 60°C, 79% RH, 24 h) (Kato et al., 1992), protamine (10:1 w/w, 60°C, 79% RH, 10 day) (Matsudomi et al., 1994) and OVA (1:1 w/w, 50°C, 65% RH, 3 week) (Kato et al., 1990) have shown poor surface properties. On the other hand, the EP of casein remained unchanged upon glycation with glucose, arabinose, lactose, ribose, or galactose (Groubet et al., 1999) under "wet" conditions (60°C, 1:1000 molar ratio protein:sugar, 6 days). More recently, however, Moreno et al. (2002) have shown that reaction of caseinomacropeptide with lactose (1:1.5 w/w, 40°C, 44% RH) improved the emulsifying activity (EA) of the native protein. Further, the increase in EA was more pronounced after 5 and 9 days storage rather than 2 days suggesting that the degree of lactosylation might be related to its EP.

Interestingly, Saeki (1997) found that glucosylation of carp myofibrillar protein improved both the EA and emulsifying stability (ES) of the native protein. Further, in concurrence with Moreno et al. (2002) maximum improvements in EP were observed for maximum decreasing rate of available lysine (46.7%). Moreover, Saeki (1998) reported that the EP of both glucosylated myofibrillar and myofibrillar-DX conjugates showed marked improvements in their EP relative to the unheated mixtures. Thus, despite earlier studies, glycation with small carbohydrates is an effective method of improving the functional properties, in particular, the emulsifying properties, of food proteins.

Affect of Initial Protein: Sugar Ratio on Emulsifying Properties

Shu et al. (1996) reported that the EP of conjugates prepared by reaction of lysozyme and GM of various MW (6–12 kDa and 24 kDa) (1:4 lysozyme:GM mole ratio, 60°C, 79% RH) were greatly increased in proportion to the length of the attached carbohydrate, whereas conjugation with an oligosaccharide from xyloglucan (XG) (1.4 kDa) (1:8 mole ratio, 60°C, 79% RH, 24 h) or GM (3.5–6 kDa) (conditions as described above) did not improve the EP. The authors concluded that GM with MW>6–12 kDa was essential for improved EP.

It is believed the unique balance of hydrophobic and hydrophilic character of the protein-sugar conjugates plays a

critical role in the mechanism behind the observed increase in EP. During emulsification the hydrophobic residues of the protein molecule are anchored in the oil droplets whereas the attached saccharides attract water molecules around the oil droplets. This serves to accelerate the formation of a thick, steric stabilizing layer around the emulsion, thereby inhibiting the coalescence of oil droplets (Khan et al., 1999).

In contrast to the findings of Shu et al. (1996), Nakamura et al. (2000) reacted lysozyme with a XG hydrosylate (1.4 kDa) under similar conditions (60°C, 79% RH) but with a vastly greater excess of oligosaccharide (1:100 mole ratio). They found that relative EA doubled within 24 h incubation, and continued to increase with reaction time. After 24 h, ES also increased and reached a steady state after 3 days. In addition, the enzymatic activity of lysozyme was sustained over the 3 days, whereas it fell dramatically to 13.3% and 78.9% in the lysozyme-DX and lysozyme-GM conjugates, respectively (Nakamura et al., 1991). Furthermore, the conjugate was rapidly formed in the oligosaccharide mixture (Nakamura et al., 2000), whereas it took more than 2 weeks in the polysaccharide system (Nakamura et al., 1992).

The disparity between the results from Shu et al. (1996) and Nakamura et al. (2000) may possibly be attributed to the difference in initial protein:sugar mole ratio used. For a given time span, it could be expected that a greater number of oligosaccharide chains attach to the protein when more of the reactant is present. The consequent increase in steric stabilization, due to the increased number of attached sugar residues, may endow greater emulsifying properties to the conjugate. The fact that the lysozyme conjugate having 2 mol of polysaccharide showed better EP than lysozyme attached with 1 mol of polysaccharide (Shu et al., 1996) appears to corroborate this theory. On the other hand, for maintenance of the biological activity of lysozyme, results suggest that simply the chain length of the saccharide alone may be the most important factor (Nakamura et al., 2000).

These results raise the question of whether there is a direct correlation between the initial sugar concentration and the increase in EP of the conjugate. The work of Dickinson and Galazka (1991) suggests the mechanism is more complex. In their study β -lactoglobulin (β -LG) was conjugated to DX (4.85 kDa) (60°C, 35–40% RH) at 1:1 and 1:3 w/w. Both conjugates showed improved EP relative to native β -LG. However, the average droplet size of β -LG-DX prepared at 1:3 w/w was larger than that of the 1:1 w/w, and hence produced a less stable emulsion. More recently, Kim et al. (2003) showed that the EP of BSA-GM (22 kDa) conjugates reacted (60°C, 79% RH) at 1:6 had better ES than conjugates reacted at 1:3, 1:9, 1:12 and 1:15 BSA:GM mole rate. Further, Akhtar and Dickinson (2003) observed that the best ES of whey protein isolate (WPI)-DX (500 kDa) conjugates (80°C, 79% RH) was achieved at WPI:DX mole ratio ~ 7 , rather than ~ 22 or 3. It seems plausible, then to propose that the initial protein:sugar mole ratio has a major influence on the EP of conjugates prepared through the Maillard reaction. It may be that, when the proportion of saccharide in the conjugate reaches a certain maximum level, the availability of the protein

to rapidly adsorb at the o/w interface during emulsification is decreased, and hence its EP is decreased.

If this is the mechanism then the extent of protein polymerization during the reaction will also affect the final EP of the conjugate. Matsudomi et al. (1995) compared the EP of two different MW complexes formed from reaction (60°C, 79% RH, 1:1 w/w) between plasma protein and GM (20–30 kDa). The complex of lower MW had high EP, while the complex having a higher MW gave EP considerably less than that of the unreacted plasma protein-GM mixture. Since in this case, the complex of high MW had a larger proportion of protein, the decrease in EP could be directly attributed to polymerisation of the protein during heat treatment, rather than an excess proportion of saccharide.

Affect of the Structure of the Reacting Saccharide on Emulsifying Properties

Kato et al. (1990, 1991, 1992, and 1993) reported that branched polysaccharides, such as GM and DX, efficiently improved the functional properties of protein-polysaccharide Maillard conjugates, while straight chain polysaccharides did not. This suggests that branched polysaccharides provide more steric hindrance, preventing coalescence of the coated fat droplets.

The ability of branched polysaccharides to improve the EP of α_s -casein, however, was not the same. After conjugation with DX (60–90 kDa) or GM (15 kDa) (1:3 w/w, 60°C, 79% RH) lysine modification was $\sim 30\%$, in each case and electrophoretic studies suggested that 3–4 lysine were bound by polysaccharide. Yet, conjugates of α_s -casein-DX, displayed slightly better EP both at pH 7.4 and in the presence of salt, whereas the α_s -casein-GM conjugate was slightly better under acidic conditions (Kato et al., 1992). In a separate study, Babiker and Kato (1998) investigated the EP of sorghum protein after conjugation with DX and GM. The reaction was performed under similar conditions (1:5 w/w, 60°C, 79% RH). Although both conjugates showed improved EP (EA increased 1.3–1.9 fold; ES increased 7–10 fold) the sorghum-GM conjugates were at the lower end of the scale in both cases. Thus, it is suggested that structural differences between branched sugars may influence the EP of their Maillard conjugates.

Affect of Reaction Time on Emulsifying Properties

Generally speaking, unheated protein/carbohydrate mixtures have similar EP to the native protein. EP then increases as the Maillard reaction proceeds (Kato and Kobayashi, 1991; Kato et al., 1990, 1991 and 1992; Khan et al., 1999; Matsudomi et al., 1994; Moreno et al., 2002; Song et al., 2002). The EP of α_s -casein-DX (60–90 kDa) and α_s -casein-GM (15–25 kDa) (Kato et al., 1992) reached a steady state after 24 h reaction (1:3 w/w, 60°C, 79% RH) whereas it took 5 days for plasma protein-GM (20–30 kDa) (1:1 w/w, 60°C, 79% RH) to reach a

steady state (Matsudomi et al., 1995) and an even longer time of 10 days for protamine-GM (20–30 kDa) (1:3 w/w, 60°C, 79% RH) (Matsudomi et al., 1994). The slow reaction of protamine-GM was attributed to the fact that protamine consists of 85% arginine with small amounts of valine, proline, and serine and has no lysine. The guanidine groups of arginine have less reactivity than free (available) amino groups (Lea and Hannan, 1950). Moreover, in contrast to caseinates, globular proteins require longer times to form protein-carbohydrate conjugates due to their compact conformation.

Interestingly, lysozyme-XG (1.4 kDa) conjugates were reported to reach a steady state in their ES after 3 days, but EA continued to increase even after 7 days of reaction (Nakamura et al., 2000). On the other hand, lysozyme-DX (1:5 w/w, 60°C, 79% RH) (Nakamura et al., 1991) and OVA-glucose-6-phosphate (1:0.94 w/w, 50°C, 65% RH) (Kato et al., 1995) showed maximum increases in their ES and EA, respectively after 2 weeks (Nakamura et al., 1991) and 1 day (Kato et al., 1995) after which further heating negatively impacted their EP.

Studies indicate, therefore, that EP can be expected to increase as the conjugates are formed. However, due to our lack of understanding of the structural attributes of the conjugates that endow them with improved functional properties, it would suggest that regularly monitoring the EP of the Maillard glycoconjugates throughout the reaction may be necessary. Further, optimal reaction times will vary depending on the reactants and the reaction conditions.

Affect of the Amount of Unreacted Lysine on Emulsifying Properties

Generally, protein conjugates that display improved EP are reported to have between 1.5–4 moles polysaccharide attached (Al-Hakkak and Kavale, 2002; Kato et al., 1990, 1992, and 1993; Khan et al., 1999; Kim et al., 2003; Nakamura et al., 1991 and 1992). On the other hand, values of lysine modification are much more variable, ranging, for example, from 25% to >50% (Al-Hakkak and Kavale, 2002; Kato et al., 1990, 1992, and 1993; Khan et al., 1999; Kim et al., 2003; Nakamura et al., 1991 and 1992). Hence, the extent to which the percent of modified lysine residues affects the functional properties of food proteins is likely to depend not only on the initial lysine content but more importantly on any critical role the residues may have in stabilizing protein structure and/or their role in the mechanism responsible for the functional properties of the protein. To illustrate, a phosvitin-GM conjugate having excellent EP had its hydrophobic N and C terminal protein segments removed. The EP were dramatically decreased as these segments were essential for anchoring the glycoconjugates to oil droplets during emulsification (Khan et al., 1999).

Affect of the Overall Net Charge on Emulsifying Properties

Darewicz and Dziuba (2001) reported the amino terminal of β -casein was critical for its ES properties. The highly charged

N-terminal of β -casein increased the electrostatic-steric repulsive forces between glycosylated β -casein molecules, preventing rapid coalescence and producing stable emulsions. On comparing the EP of lysozyme-GM and α -lactalbumin (α -LA)-GM prepared at 60°C, 79% RH for 2 weeks at 1:1 w/w, Nakamura et al. (1994) showed that both conjugates showed a similar decrease (~25%) in available lysine, and although their EA were almost the same and much improved over the native protein, the ES of α -LA was not so remarkably improved by conjugation with GM as that of lysozyme. Acetylation of unreacted lysine residues in the lysozyme conjugate showed that the positive charge on the protein (contributing to electrostatic repulsion) was necessary for its remarkable improvement. Based on electrophoretic studies, the authors suggested that ~3 and ~2 moles of GM bound to α -LA and lysozyme, respectively.

To investigate the effect of the charge of the polysaccharide, Song et al. (2002) reacted lysozyme with the cationic carbohydrates, low MW chitosan (LMW, 3–30 kDa), and high MW chitosan (HMW, 400 kDa) (60°C, 79% RH), and with the neutral polysaccharide, GM (15 kDa). Compared to conjugation with GM, ES of lysozyme at pH 4 was greatly improved by conjugation with chitosan, particularly for HMW chitosan. The authors suggested that the number of cationized groups in chitosan plays a key role in inhibiting the coalescence of oil droplets to produce a stable emulsion. Similar findings have been reported for β -LG-chitopentaose conjugates (50°C, 79% RH, 27 h) (Hatorri et al., 2000). Unlike conjugation with LMW glucosamine, conjugation with chitopentaose dramatically improved the relative EA of β -LG under acidic conditions and in the presence of salt. The high EA of chitopentaose was attributed to its high net charge, which had a protective effect against shielding of the electrical charge in the emulsifier.

Conjugation of proteins with anionic carbohydrates via the controlled Maillard reaction is reported to have the same effect on protein structure as succinylation. Succinylation is a chemical modification that converts cationic amino groups to anionic residues. The alteration of charge induced by succinylation is reported to be responsible for the improved functional properties of the protein. Using the natural Maillard approach, OVA was reacted with the anionic carbohydrate, glucuronic acid (GlcUA) (50°C, 65% RH, 3 days) (Aoki et al., 1999). Despite a marked improvement in the EA of OVA, the carboxyl group of GlcUA markedly promoted the advanced stages of the Maillard reaction. This rapidly induced protein polymerization and adverse browning development, which intensified with an increase in incubation time. Reaction times of less than 1 day were essential to avoid these adverse effects. Similar findings were reported on reaction of OVA with glucose-6-phosphate (50°C, 65% RH, 3 days) (Kato et al., 1995). On the other hand, prolongation of the chain length depressed the browning and protein polymerization (Aoki et al., 2001). Thus, reaction of β -LG and alginic acid (50°C, 79% RH, 169 h) (Hatorri et al., 1996) or alginic acid oligosaccharide (50°C, 79% RH, 24 h) (Hatorri et al., 1997) improved EP of the protein without causing undesirable side

effects. The improvement in EP was attributed to the high net charge of alginic acid.

Soybean protein isolate was conjugated with the anionic polysaccharide carboxymethyl cellulose (CMC) (60°C, 79% RH, 5 weeks) using a low, medium, and high viscosity CMC (Diftis and Kiosseoglou, 2003). Conjugation improved EP of soybean protein. Similar trends were seen for all protein-CMC conjugates and improvements were irrespective of heating for 3 or 5 weeks. All conjugates, however, were light brown in color and showed evidence for protein polymerization after just 1 week incubation and increased thereafter. Hence, even reaction with polysaccharides can induce browning and protein polymerization. In fact, Kato et al. (1990) described OVA-DX (60°C, 65% RH, 1:5 w/w, 3 weeks) conjugates as having considerable browning.

Such studies, however, suggest that conjugation of proteins with both anionic and cationic saccharides, through the Maillard reaction, can effectively improve the functional properties, particularly EP of proteins. Reaction with charged oligo- or polysaccharides, however, is preferred over shorter chain length saccharides, to avoid discoloration and protein polymerization, associated with the advanced Maillard reaction.

SOLUBILITY

In contrast to the classical studies of Kato Y et al. (1986 and 1988), Lea and Hannan (1950), and Mohammed et al. (1949) which show a marked decrease in protein solubility, recent studies have shown a significant improvement in protein solubility following glycation. Although it has been suggested that the improved solubility may be attributed to a limited degree of glycation, this cannot fully account for these discrepancies. One must also consider the possibility of disulfide bonding, and isopeptide cross-linking, for example. Thus, the restricted attachment of hydrophilic sugar residues to proteins must also presumably reduce the propensity of the protein to self-interact. The biochemistry of the protein and the molecular structure and chemistry of the reacting sugar will therefore also be important factors.

For example, Saeki and Inoue (1997) prepared water-soluble fish myofibrillar proteins by conjugation with glucose through the Maillard reaction (1:9 w/w, 40°C, 65% RH, 12 h). The solubility improvement of myofibrillar protein occurred when just 17% of the available lysine residues were glycated. As expected, a longer incubation time (24 h) was required at 35°C, during which 21% of the reactive lysine in myosin were modified with glucose (Tanabe and Saeki, 2001). On the other hand, the same effect of glycosylation was obtained after 2 h (35°C) on reaction with ribose (Tanabe and Saeki, 2001). However, for glycosylation of scallop myofibrillar proteins, a 60% decrease in reactive lysine was required to achieve the solubility improvement (Katayama et al., 2002). Further, in contrast to its reaction with small reducing sugars, Sato et al. (2000) reported that fish myofibrillar protein glycated with alginate oligosaccharide affected the same solubility improvement with only a small (<10%) de-

crease in lysine. Hence, by using a different reducing sugar, the same increase in functionality can be obtained and the substantial decrease in lysine content avoided.

Saeki (1997) showed that, despite a 50% decrease in reactive lysine, there was little effect on the solubility of carp myofibrillar incubated in the presence of glucose. Further, no discoloration was observed for the heated protein-glucose mixture and the EP of the protein were considerably improved. In contrast, myofibrillar protein alone showed considerable loss of solubility on lyophilization with further decrease on incubation.

Although glycation was essential for improving the EP of myofibrillar protein, solubility of the protein was also maintained after incubation in the presence of sorbitol. Hence sorbitol served to protect the protein during the stresses induced by freezing and freeze-drying and thereby maintain its structural integrity during subsequent heat treatment.

Shepherd et al. (2000) showed that caseinate-maltodextrin (1.4 kDa) conjugates (1:1 w/w, 60°C, 79% RH, 120 h) had high solubility at all pH values. In contrast the caseinate control was minimally soluble near its isoelectric point, pH 4.0–4.5. Extensive cross-linking was presumably prevented by the lack of any significant degradation of the Amadori adduct. Substitution of maltodextrin at the C-4 hydroxyl group of the glucosyl residues makes it less energetically prone to post-Amadori Maillard reactions (Kato A et al., 1988). Thus, the advanced stages of the Maillard reaction were largely prevented. The improvement in solubility was observed within just 5 h of incubation and the reaction was largely complete within 24 h. EP at pH 4.8 were also improved. Between pH 6–9 however the EP of the unheated mixture and conjugate were very similar. Therefore the primary effect of conjugation was an improvement in solubility of the caseinate emulsifier rather than any localized improvement in EP due to steric stabilization or polysaccharide entanglement. The authors suggested that the functional properties of caseinates might be further improved by its reaction with maltodextrin of higher (>1.4 kDa) MW. However, no data addressing this concept have since been published.

Groubet et al. (1999) showed solubility of β -casein was improved by heating aqueous solutions of the protein with various low molecular weight reducing sugars under anaerobic conditions, at 60°C for 3 days. Modification levels ranged from 20–30% with lactose, glucose and galactose, to 40–70% with ribose and arabinose. In each case the conjugates showed improved solubility between pH 4.5–6.0. In the absence of oxygen, production of reactive glycoxidative products formed during the initial stages of heating was minimized, and hence the later stages of the reaction, such as protein cross-linking, occurred less rapidly (Thornalley et al., 1984; Yaylayan and Hughues-Despointes, 1994; Yeboah et al., 2000).

FUTURE WORK

The Maillard reaction is one of the few ways of creating covalent bonds between food components using a food-grade

approach. Moreover, as exemplified in this review, there is substantial evidence to show that glycation via the Maillard reaction can improve many important functional attributes of food proteins. More work is needed, however, to develop industrially feasible methods for preparing the Maillard glycoconjugates with optimum functionality in real food systems. Further research is also needed to provide a better understanding of the relationship between the structure of the conjugates and their functional properties.

It is an exciting area of research, which holds great potential and promising rewards. With greater understanding we expect that Maillard-derived glycoconjugates will emerge on the market as functional food ingredients having designer functionality.

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