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Food Chemistry 79 (2002) 343–349

Food Chemistry

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Maillard crosslinking of food proteins I: the reaction of glutaraldehyde, formaldehyde and glyceraldehyde with ribonuclease

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Received 22 January 2002; accepted 7 March 2002

Abstract

The Maillard reaction influences not only the colour and flavour of foods, but also their texture, via protein crosslinking. In this, the first of a series of three papers to assess the potential of the Maillard reaction to alter food properties in situ, the capacities of three molecules—formaldehyde, glyceraldehyde and glutaraldehyde—to crosslink a model protein—RNAse—were compared. All three molecules crosslinked RNAse in vitro, with glutaraldehyde reacting at a much greater rate than either glyceraldehyde or formaldehyde, whose rates were comparable. Crosslinking correlated well with loss of lysine in the proteins for glutaraldehyde and glyceraldehyde, with some anomalies for formaldehyde. The differences in reactivity are explained by the proposed mechanisms of crosslinking for each of the three molecules. An understanding of the range of reactivity of crosslinking molecules, and how this relates to their molecular structure, may help us understand how to harness the Maillard reaction during food processing.

Keywords: Protein crosslinking; Maillard; Glycation; Food texture; RNAse

1. Introduction

Three key properties of food to which consumers respond — colour, flavour and texture — are all influenced by the Maillard reaction, but the last of these — texture — has received the least attention (Easa, Hill, Mitchell, & Taylor, 1996; Pellegrino, van Boekel, Gruppen, Resmini, & Pagani, 1999). Previous work in our laboratory has established unequivocally that protein crosslinking can profoundly affect the texture of food, specifically bread and croissants (Gerrard et al., 1998, 2000). Furthermore, the Maillard reaction has been shown to effect protein crosslinking on a timescale relevant to food processors (Fayle, Gerrard, Simmons et al., 2000). In this paper, we explore the potential of three molecules known to crosslink proteins — formaldehyde, glyceraldehyde and glutaraldehyde — by assessing their relative activities with a model food protein, RNAse. In the accompanying papers, we extend the study to wheat proteins, in vitro and in situ.

The three molecules (see Fig. 1) were selected since each has been reported to crosslink proteins, but all have different structural features. Glutaraldehyde is, perhaps, the best known of all protein crosslinking molecules, although the mechanism of reaction is still not clearly established (Ford & Pesce, 1981; Hermanson, 1996; Monsan, Mazarquil, & Puzo, 1975). The crosslinking ability of formaldehyde is also well-documented (Galembeck, Ryan, Whitaker, & Feeney, 1977; Singh, 1991) although less has been said of the mechanism of this reaction. For example, it remains uncertain as to whether a single molecule crosslinks two protein chains (Marquie, Tessier, Aymard, & Guilbert, 1997), or whether the chemistry is more complicated, particularly given the complex self-condensation reactions that formaldehyde is known to undergo (Matsumoto, Yamamoto, & Inoue, 1984). Neither glutaraldehyde nor formaldehyde are appropriate for use in food; they do, however, provide elegant models for molecules possessing one or two reactive carbonyl moieties.

Glyceraldehyde is a known component of foods, and has recently attracted attention as a molecule that is reactive during Maillard browning (Keller, Widzicha,

Abbreviations: RNAse A, ribonuclease A; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; OPA, *o*-phthaldialdehyde.

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0308-8146/02/$ - see front matter
PII: S0308-8146(02)00174-7

Leong, & Berger 1999). As a simple sugar, this molecule was included as a compound that is more likely to be found in foods, with more complex structural features than either glutaraldehyde or formaldehyde. It has previously been reported to crosslink proteins (Acharya, Cho, & Manjula 1988; Lee, Simpson, & Ortwerth 1999; Prabhakaram & Ortwerth, 1994), most recently being implicated in the formation of pentosidine (Chellan & Nagaraj, 2001).

2. Materials and methods

Unless otherwise stated, all materials were obtained from Sigma Chemical Company (St Louis, MO, USA). RNAse was Type XII-A from bovine pancreas.

RNase A (25 mg/ml) was incubated at 37 °C with a variety of concentrations, up to 200 mM, of carbohydrate at a starting pH that had been adjusted to 7.0, as determined by pH paper. For glutaraldehyde incubations, samples were removed hourly for 5 h. This was extended to periodic sampling up to 48 h for formaldehyde and glyceraldehyde incubations. Samples were stored at −80 °C. A zero-time sample and an RNase A frozen control were placed immediately in liquid nitrogen and stored at −80 °C. An RNAse A control was also incubated for the duration of the experiment.

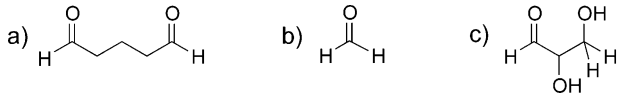

Fig. 1. The structure of (a) glutaraldehyde, (b) formaldehyde and (c) glyceraldehyde.

Protein crosslinking was monitored by previously published electrophoretic methods (Fayle, Healy, Brown et al., 2001; Fayle et al., 2000). Lysine availability was monitored by a modification of the OPA method of Bertrand-Harb (Fayle et al., 2001). Protein concentrations were estimated by the method of Bradford (Bradford, 1976).

All experiments were performed in triplicate to ensure reproducibility.

3. Results and discussion

3.1. Choice of model system

Previous investigations in this and other laboratories have established that RNAse A is an excellent model protein for crosslinking studies; this is attributed to its relatively small size (13,680 Da), making oligomerisation of the monomer easy to detect. It is also thermally and chemically stable, and is readily available from commercial suppliers. RNAse A was therefore chosen, as the model protein, for the examination of the crosslinking ability of each compound in this study, prior to the analysis of the less well-characterised wheat proteins (see following paper).

RNAse A contains 11 free amino groups, originating from the ε-amino groups of 10 lysine residues, and a

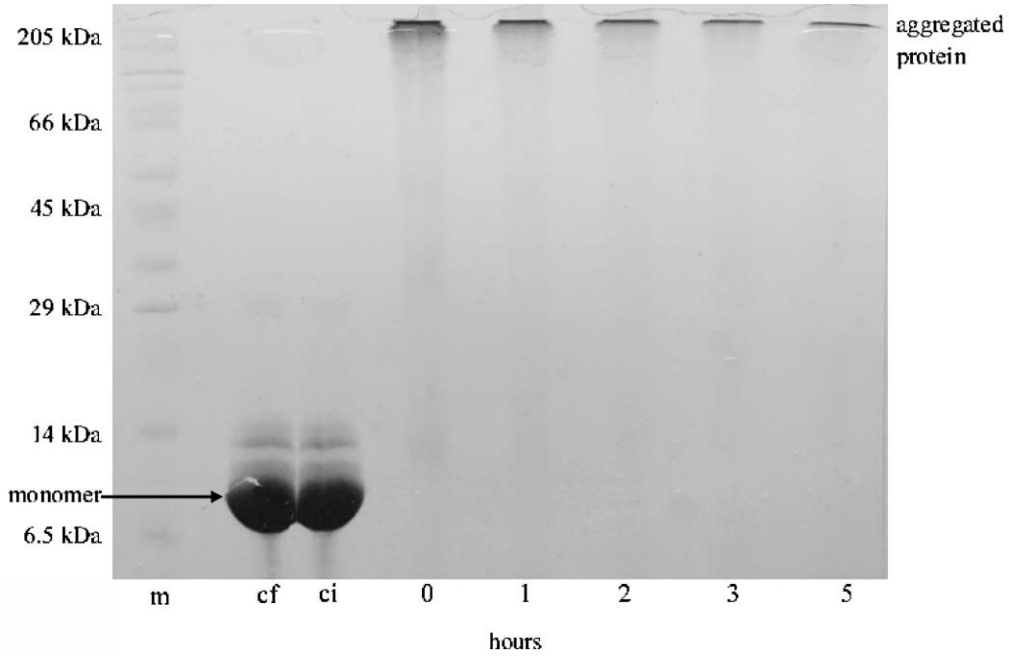

Fig. 2. Typical SDS-PAGE gel of RNase A incubated in aqueous solution with 200 mM glutaraldehyde at 37 °C. The frozen control (cf) and incubated control (ci) appear in monomeric form. Addition of glutaraldehyde causes the immediate disappearance of monomeric RNase A, with the formation of aggregates greater than 205 kDa in size, which are unable to enter the resolving gel. m = Wide range SigmaMarker.

terminal α-amino group. Previous work, using OPA analysis, showed that nine of these lysine residues were reactive (Fayle et al., 2001) and that protecting these lysine residues, prior to incubation with a series of carbonyl compounds related to those used in this paper, prevented the reaction (Fayle et al., 2000). We thus elected to monitor the crosslinking of RNAse using both electrophoretic analysis and lysine counting methods.

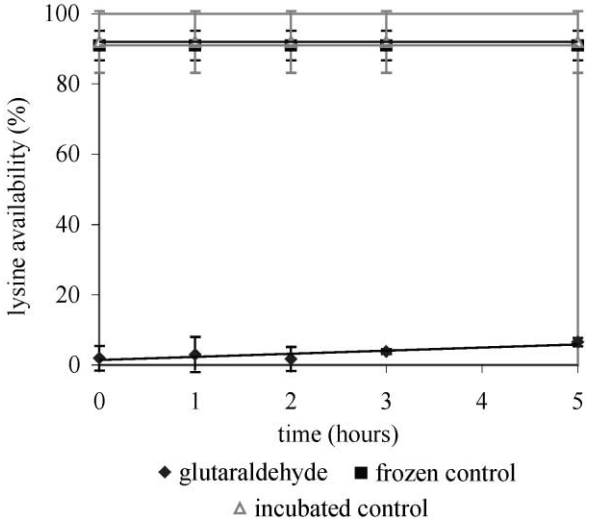

Fig. 3. Typical lysine analysis of RNase A incubated with 50 mM glutaraldehyde at 37 °C. Lysine availability (100%) was determined using an RNase A standard curve. Each point represents the mean of triplicate measurements. Error bars represent the standard error of the mean.

3.2. Incubations with glutaraldehyde, formaldehyde and glyceraldehyde

Crosslinking studies, using RNAse as a model protein, confirmed the well-established result that glutaraldehyde crosslinks proteins almost instantaneously. Reaction was accompanied by a yellow coloration, whilst the controls remained colourless. Fig. 2 clearly illustrates the complete aggregation of RNAse on addition of 200 mM glutaraldehyde. The crosslinking was accompanied by a dramatic loss in the availability of all measurable lysine residues in the protein, confirming that the crosslinking mechanism involves reaction of these amino groups. This is shown in Fig. 3. The data suggest that all available lysines are employed in protein crosslinking by glutaraldehyde.

The incubation of glyceraldehyde with RNAse A resulted in protein crosslinking at a considerably slower rate than that observed with glutaraldehyde. The SDS-PAGE results are shown in Fig. 4 and show the presence (traces) of dimer in the control samples that was not removed by the reducing conditions of the electrophoresis. This has been noted in previous studies using RNAse (Picolli, Tamburrini, Piccialli et al., 1992; Liu, Gotte, Libonati, & Eisenberg, 2001). The intensity of the dimer band increases throughout the incubation, with trimer formation occurring after 1 h. After 48 h some protein is too large to enter the gel, and a large degree of smearing is evident, consistent with the formation of crosslinked protein of varying sizes. Lysine analysis

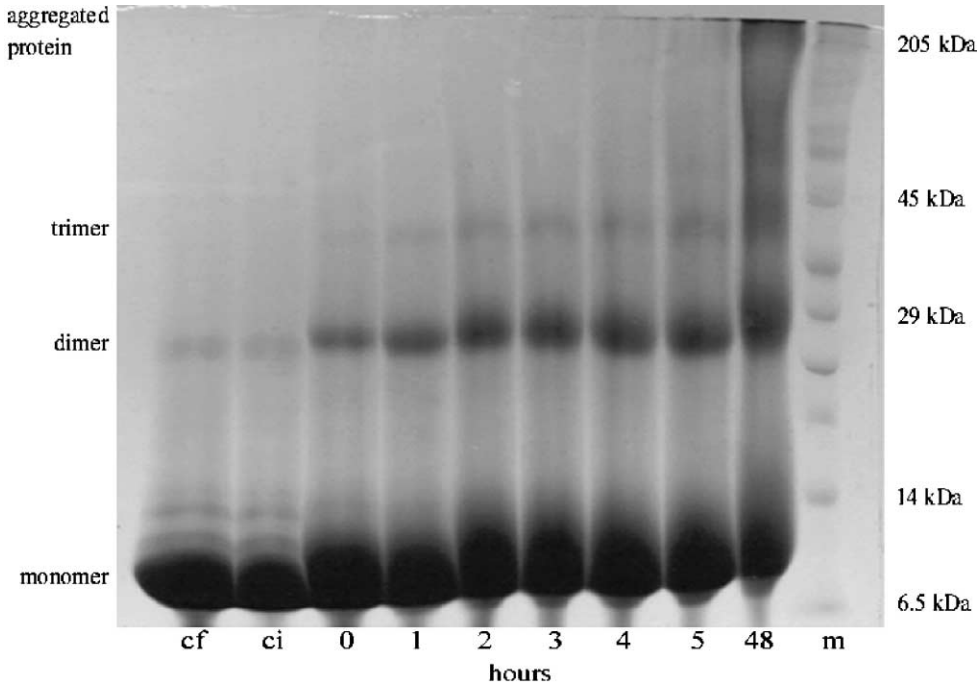

Fig. 4. Typical SDS-PAGE gel of RNase A incubated in aqueous solution with 200 mM glyceraldehyde at 37 °C. The frozen control (cf) and incubated control (ci) appear largely in monomeric form, with some dimer present. Addition of glyceraldehyde causes dimerisation to increase throughout the incubation period, with trimer formation after 1 h. m = Wide range SigmaMarker.

(Fig. 5) shows a significant decrease in available lysine, although the extent of lysine loss is substantially less than for glutaraldehyde (Fig. 3), reflecting the lower reactivity of glyceraldehyde.

Incubation with formaldehyde and subsequent analysis suggested that formaldehyde crosslinks RNAse A at a slightly slower rate than glyceraldehyde. Fig. 6 shows that the RNAse dimer band increases in intensity over time and, after 1 h, trimer formation can be seen. The concentration of protein in the trimer form, however, appears to be lower than that observed in Fig. 4, and there is less smearing of the sample along the length of the lane at 48 h. It can be seen in Fig. 7 that the lysine availability of RNAse A, when incubated with formaldehyde,

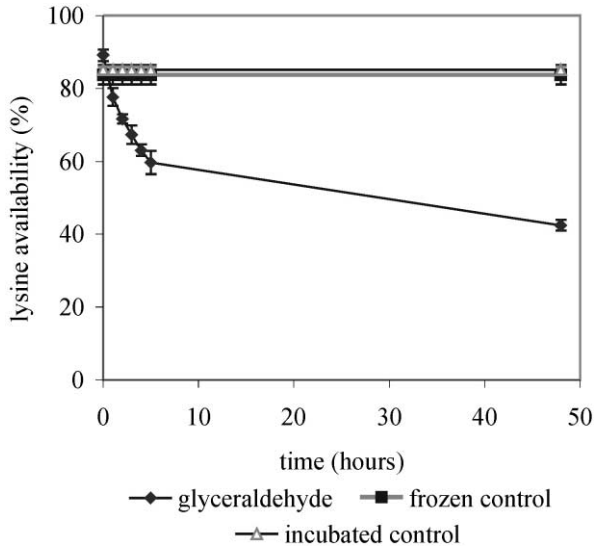

Fig. 5. Typical lysine analysis of RNase A incubated with 50 mM glyceraldehyde at 37 °C. 100% lysine availability was determined by an RNase A standard curve. Each point represents the mean of triplicate measurements. Error bars represent the standard error of the mean.

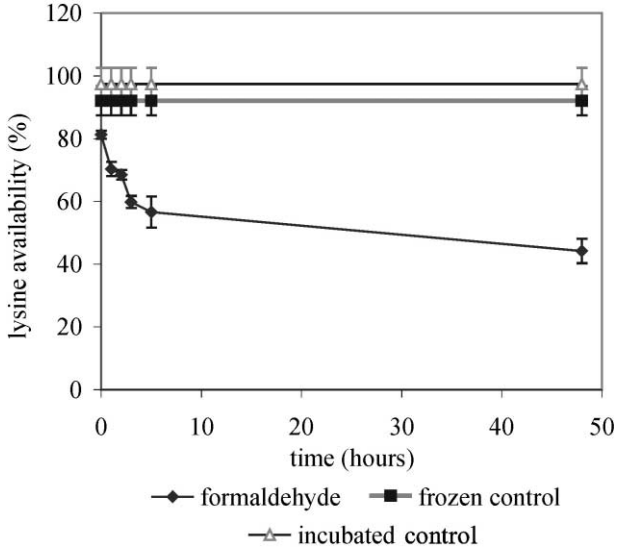

Fig. 7. Lysine availability of RNase A incubated with formaldehyde at 37 °C. 100% lysine availability was estimated as the absorbance of 25 mg/ml as determined using an RNase A standard curve. Error bars represent the standard error about the mean of triplicate values.

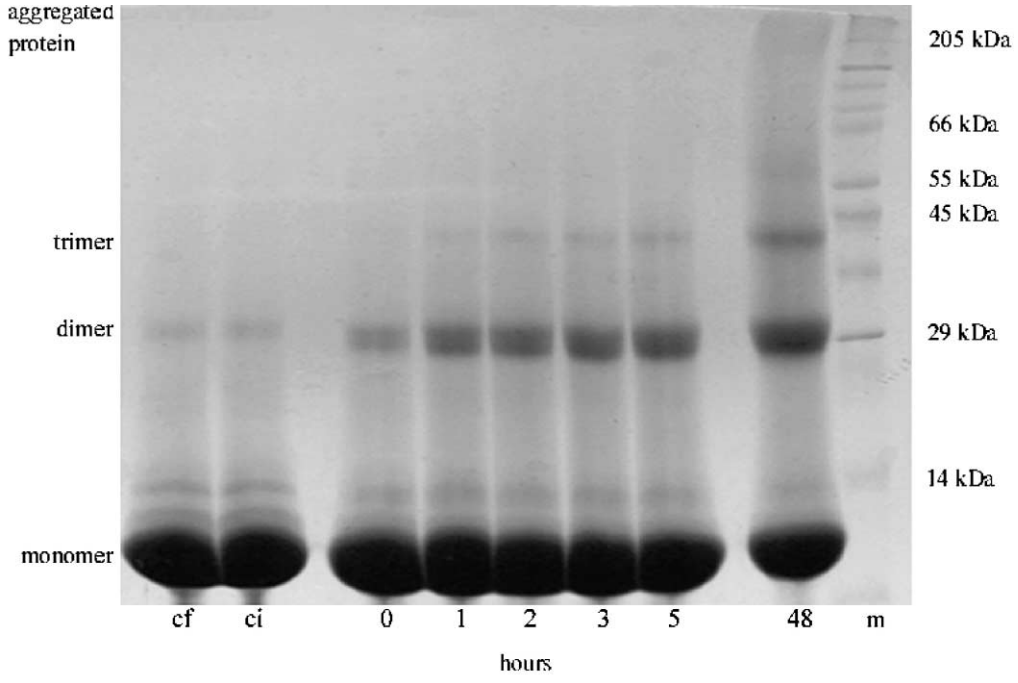

Fig. 6. Typical SDS-PAGE gel of RNase A incubated in aqueous solution with 200 mM formaldehyde at 37 °C. The frozen control (cf) and incubated control (ci) appear largely in monomeric form, with some dimer present. Addition of formaldehyde causes the dimer band to intensify throughout the incubation period, with trimer formation after 1 h. m = Wide range SigmaMarker.

drops immediately from almost 100% in the controls, to approximately 80%, and continues to decrease steadily during the first 5 h. After 48 h, lysine availability is analogous to that after the equivalent incubation period for glyceraldehyde. It would be interesting to ascertain whether this represents reaction of specific lysines on the protein surface. Despite appearing to crosslink RNAse A at a slower rate than glyceraldehyde, the decrease in lysine availability is very similar to that observed for the glyceraldehyde incubations. This suggests that reaction

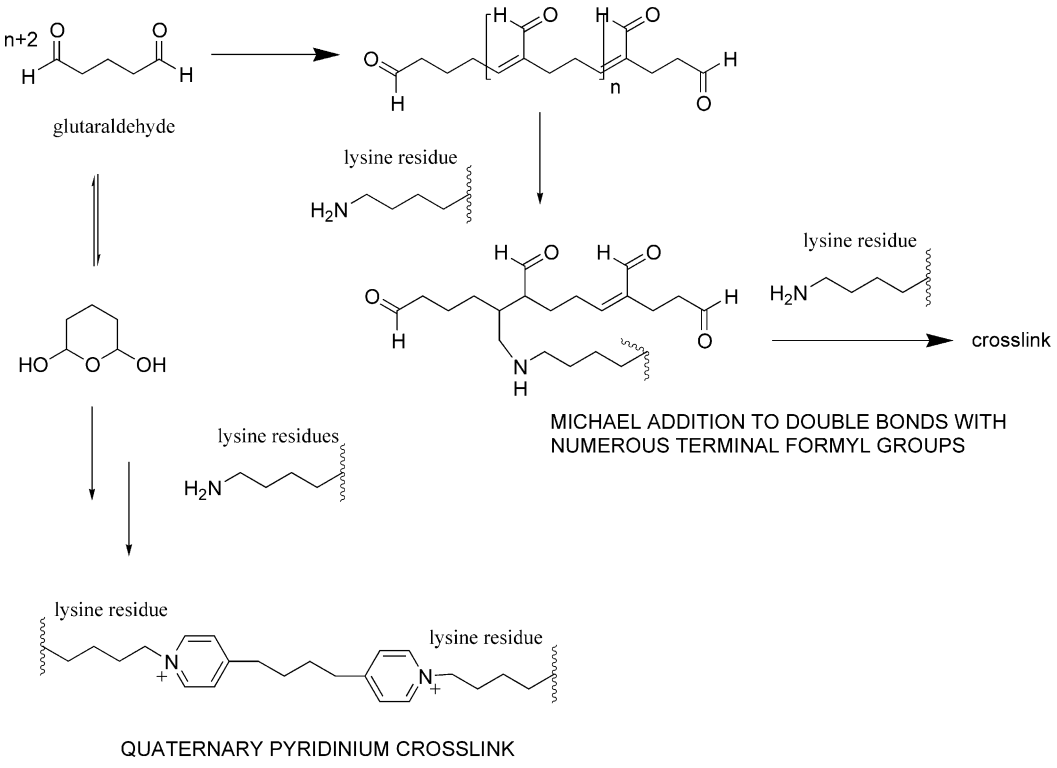

Fig. 8. Proposed mechanisms for crosslinking of proteins by glutaraldehyde via the Maillard reaction, adapted from Hermanson (1996).

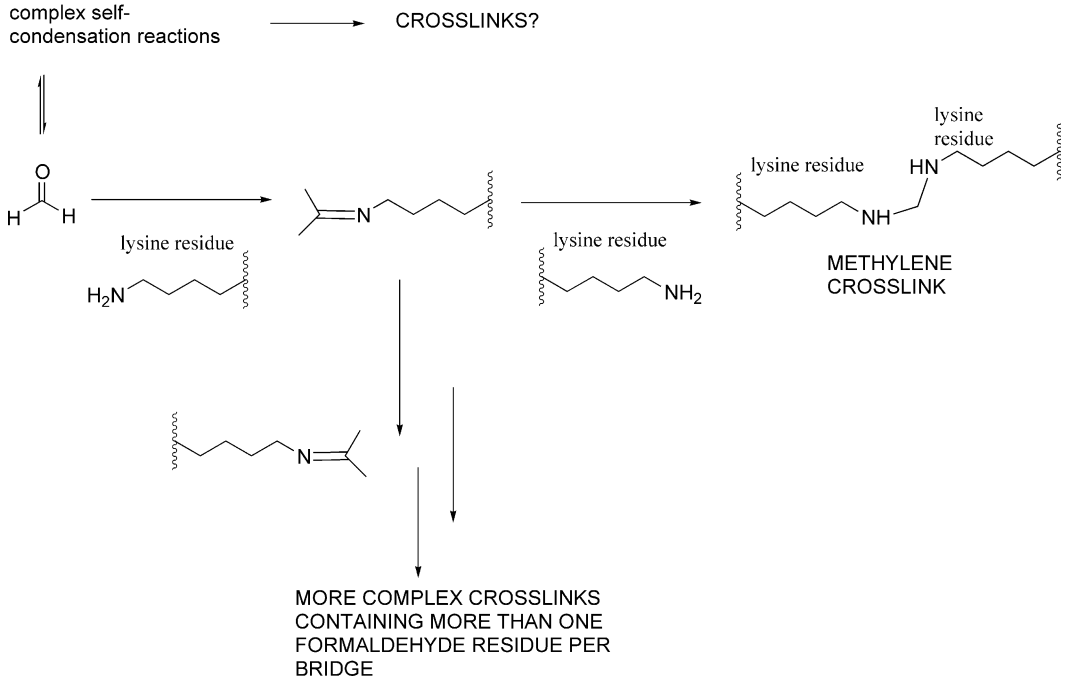

Fig. 9. Proposed mechanisms for crosslinking of proteins by formaldehyde via the Maillard reaction.

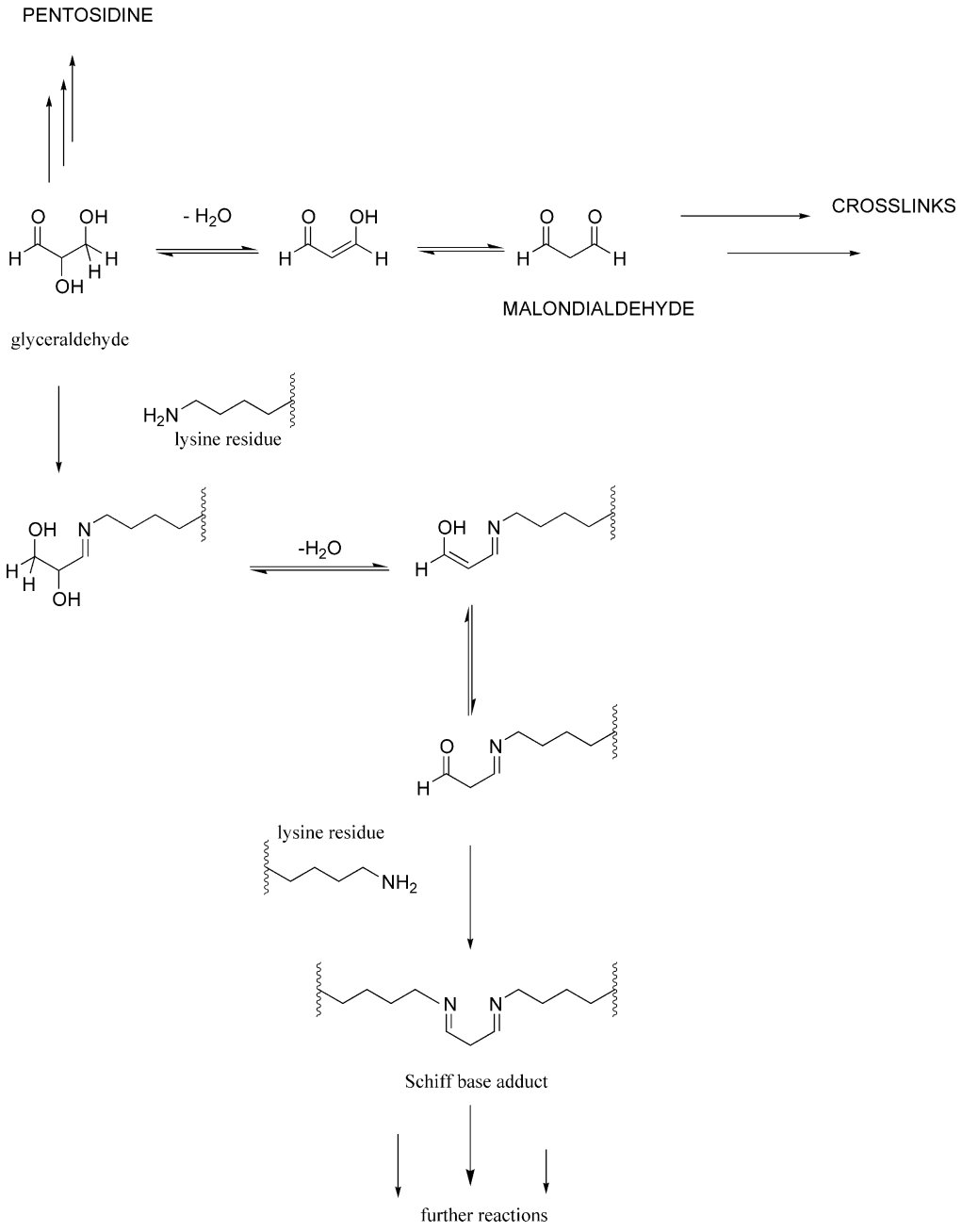

Fig. 10. Proposed mechanisms for the crosslinking of proteins by glyceraldehyde via the Maillard reaction.

with lysine residues is not the rate-determining step of the crosslinking process, which must therefore reflect subsequent chemistry.

The ratio of rates of the formaldehyde and glutaraldehyde loss of lysine availability is consistent with the data of Marquie et al., who measured the lysine contents of protein films created by crosslinking cottonseed proteins (Marquie et al., 1997; Marquie, Tessier, Aymard, & Guilbert, 1998). The order of crosslinking activity, with glutaraldehyde far more reactive than the other two molecules, is not surprising, since glutaraldehyde is a dicarbonyl compound with two reactive moieties, whereas direct crosslinking by formaldehyde or glyceraldehyde, with only one carbonyl group per molecule, is less likely.

4. Conclusion

The mechanisms of the protein crosslinking reactions remain open to speculation. Hermanson (1996) offers the most recent summary of glutaraldehyde crosslinking, as summarised in Fig. 8. That the crosslinking process is so much faster for glutaraldehyde than for all other molecules tested in this and other studies suggests that the chemistry is specific to this molecule, and perhaps

provides support for the quarternary pyridinium crosslink, inaccessible by smaller molecules.

Formaldehyde crosslinking has been attributed to simple methylene bridges (Marquie et al., 1997), with each crosslink therefore comprising one formaldehyde molecule per two lysines. The fact that the rate of crosslinking and the rate of lysine loss do not precisely correlate (for example, the sudden loss of lysine is not accompanied by a sudden surge in crosslinking) would seem to suggest that other mechanisms may be operating in addition to that suggested, as summarised in Fig. 9.

Combining the suggested mechanisms for glutaraldehyde and formaldehyde leads to a host of possible routes for the crosslinking reaction of glyceraldehyde with proteins. Perhaps the most specific of these involves the generation of malondialdehyde, or its equivalent protein bound adduct. Malondialdehyde has been shown to be an active crosslinking agent in other contexts (Miyata, Inagi, Asahi et al., 1998). These possibilities are summarised in Fig. 10.

In summary, glutaraldehyde, formaldehyde and glyceraldehyde provide a range of protein crosslinking activites and have been shown to crosslink a model protein, with implied involvement of the lysine residues. The results of these model studies will be validated for wheat proteins in vitro and in situ in the following paper.

Acknowledgements

This work was funded by Crop and Food Research Ltd as part of contract C02X0001 for the New Economy Research Fund, Foundation for Research Science and Technology, New Zealand. We thank Jackie Healy for invaluable technical expertise.

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