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Crosslinkage of proteins by dehydroascorbic acid and its degradation products

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Abstract

Protein crosslinking can have a profound effect on the structure and function of proteins in food. Dehydroascorbic acid (DHA) has been shown to be involved in Maillard type chemistry that leads to protein crosslinking. In this study, the effect of temperature on the rate of this reaction was studied. The reaction was shown to proceed rapidly at temperatures that may be encountered during food processing. In order to assess the relative reactivity of DHA and its breakdown products, five known degradation products were reacted with protein and their crosslinking ability, via Maillard chemistry, was assessed. Oxalic acid did not effect protein crosslinking. Threose, glyoxal, diacetyl and methyl glyoxal all reacted faster than DHA. The main crosslinking reaction observed was shown to involve a lysine residue. Our results suggest that these molecules may be important in determining the modification of protein functionality during food processing.

Keywords: Dehydroascorbic acid; Crosslinking; Maillard reaction; Ribonuclease

1. Introduction

Covalent crosslinks can have a profound effect on the structure and function of a protein in biological systems. The appropriate degree of crosslinkage is critical for maintaining the correct degree of firmness or elasticity for a particular cell, tissue or organ (Feeney & Whitaker, 1988). Inappropriate crosslinks, such as those found in ageing tissue and in diabetics, are increasingly associated with certain disease states (Drickamer, 1996). The importance of protein crosslinks in food systems is less well studied, but it is clear that such specific modifications of the properties of a protein are, potentially, of great practical importance in the food industry (e.g. Hill & Easa, 1998; Singh, 1991). The deliberate modification of food proteins in situ, during processing, may prove to be a valuable tool for the manipulation of food properties.

The baking industry has been chemically modifying dough proteins for many years, resulting in improved consistency, texture and strength of many baked goods (Tsen, 1965). The addition of oxidising agents has been found to improve the performance of doughs. The mechanism of this improvement is thought to be due to the formation of disulfide crosslinks between proteins (Lillard, Seib & Hoseney, 1982). We have recently shown that introducing other crosslinks into baked products can improve their properties (Gerrard, Fayle, Sutton, Newberry, Ross & Kavale, 1998; Gerrard, Fayle, Wilson, Newberry, Ross & Kavale in press).

Ascorbic acid is commonly used as a flour improver in the baking of bread: its addition to a dough improves the elasticity and gas-retaining properties of the dough and results in a larger loaf with improved texture (Lillard et al, 1982). Ascorbic acid is an unusual flour improver in that it is a reducing agent. The action of ascorbic acid as a flour improver has therefore been generally attributed to its first stable oxidation product,

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Abbreviations: RNAse A, ribonuclease A; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SE-HPLC, size exclusion high performance liquid chromatography; DHA, dehydroascorbic acid; OPA, *o*-phthaldialdehyde;

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dehydroascorbic acid (DHA) (See Fig. 1). Since interconversion occurs readily under conditions found in dough, addition of either ascorbic acid or DHA may often result in the same effect (Collins, Little & Pritchard, 1991).

The redox chemistry of DHA has been widely studied in cereal chemistry, and has generally overshadowed other possible reactions. Model studies of DHA with proteins have demonstrated that DHA can crosslink proteins, at 37°C, by mechanisms that do not involve disulfide bonding (Gerrard, Fayle, Sutton & Pratt, 1998). The mechanism is thought to involve the Maillard reaction, a general term used to describe a complex series of reactions between reactive carbonyl groups and free amine groups, such as those found on proteins (Maillard, 1912).

In the work presented here, we explore whether these Maillard reactions are likely to be significant at temperatures approaching those encountered during food processing, by investigating the effect of temperature on the reaction between DHA and the model protein RNAse A. In addition, since DHA is not a stable molecule and has many reported breakdown products (Vernin, Chakib, Rogacheva, Obretenov & Paranyi, 1998), we investigate the relative rate of the DHA crosslinking reaction compared with that of a number of its degradation products.

2. Materials and methods

2.1. Reagents

Unless otherwise stated, all materials were obtained from Sigma Chemical Co. Ribonuclease A (RNAse A) was type XII-A (from bovine pancreas). DHA was prepared by the method of Ohmori, Higashioka and Takagi (1983). Purity of the resulting product was assessed by ^{1}H and ^{13}C NMR (recorded on a Varian Unity 300 spectrometer at 300 and 75 MHz, respectively).

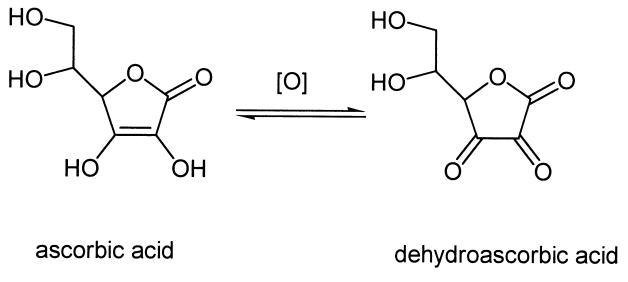

Fig. 1. Redox equilibrium between ascorbic acid and DHA.

2.2. Incubations

Unless otherwise stated, incubations were carried out in aqueous solution, at either 37, 50, 75, or 90°C, at a protein concentration of 25 mg/ml and a DHA concentration of 25 mg/ml. A 27.5 mg sample of RNAse A was dissolved in 1.1 ml of distilled water and stored on ice. Two 100 μl aliquots were removed and acted as controls, one incubated and the other stored frozen. The remaining 900 μl of protein solution was transferred to an Eppendorf tube containing 22.5 mg of DHA. After thorough mixing, the solution was equally divided into nine tubes and placed in an incubator. A tube was removed hourly, for the first 7 h, then at 10, 12 and 24 h. Samples were stored at −10°C prior to analysis by SE-HPLC. Incubations with breakdown products were carried out in an analogous fashion.

Alternatively, a 15 mg sample of protein was dissolved in 600 μl of distilled water and stored on ice. Two 100 μl controls were removed as described above. The remaining 400 μl of protein solution was thoroughly mixed with 10 mg of sugar and equally divided into four tubes and placed in an incubator, at a temperature of 37°C. Tubes were stored at −10°C prior to analysis by SDS-PAGE.

2.3. Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was carried out according to standard methods (Dunn, 1989). The samples were reduced before loading onto a 3.5% stacking gel with a 12.2% resolving gel. Samples were electrophoresed at 4°C at a constant current of 30 mA until the bromophenol blue dye had reached the lower edge of the gel, ~4.5 h. Visualisation of proteins was achieved using Coomassie Brilliant Blue.

2.4. SE-HPLC

Analyses were carried out using a Waters HPLC system consisting of a Waters 626 solvent delivery/control system with a Waters WISP717-plus automatic sampler injector and a Waters 996 diode array detector. The column, a Phenomenex Biosep SEC-4000 (300×7.8 mm), was fitted with a matching guard column (75×7.8 mm) and was maintained at 25°C. The solvent used was 1:1 water/acetonitrile containing 0.1% (v/v) trifluoroacetic acid at a flow rate of 0.5 ml/min. The column was calibrated using standards of known molecular mass (namely 13.7, 14.4, 20.1, 30, 43, 67 and 94 kDa). The injection volume was 20 μl, and a data acquisition time of 35 min was used. Spectral data were recorded over the wavelength range of 190–600 nm. The instruments were controlled and data recorded using Waters Millennium 2010 software (version 2.15) operating on a personal computer.

2.5. Protection of lysine residues (Hollecker & Creighton, 1980)

A 1 mg/ml aqueous solution of RNAse A was adjusted to pH 7.0 by the addition of the required amount of 1.0 M sodium hydroxide. Twelve aliquots of succinic anhydride (2 mg) were added with constant stirring, over a period of 6 h, during which the pH was maintained at 7.0 by the dropwise addition of 0.5 M sodium hydroxide. The resulting solution was dialysed against stirred distilled water and then concentrated by freeze-drying. Chemically reactive residues were quantified using the modification of the OPA method of Bertrand-Harb, Nicolas, Dalgalarrondo and Chobert (1993).

3. Results and discussion

3.1. Selection of a model protein system

RNAse A, a small (13.7 kDa), thermally and chemically stable protein, had previously been selected as the protein component for our ongoing model reactions, as it consistently gave the best results (Fayle, 1998). It contains 11 free amino groups corresponding to the ε-amino group of the 10 lysine residues and the single terminal α-amino group. Due to the hydrophilic nature of the amino group, each of the lysine residues is located on the surface of the folded protein (Boqui, Coll, Cuchillo & Fita, 1994). Derivatisation with OPA (Bertrand-Harb et al., 1993) showed that nine of the amino groups are chemically reactive. Some dimer was present in the control that was not removed by the reducing conditions of the electrophoresis. This has been noted in previous studies using ribonuclease (Picolli, Tamburrini, Piccialli, D. Donato, Parente & D'Alessio, 1994).

3.2. Relative rate of the DHA-mediated crosslinking reaction

RNAse A was incubated with DHA at a variety of temperatures, ranging from 37 to 90°C. Visual inspection of the subsequent reaction mixtures showed a rapid increase in the presence of coloured products, with respect to both time and temperature, consistent with previous results (Fayle, 1998; Gerrard, Fayle, Sutton & Pratt, 1998, 1999).

Due to the complex nature of Maillard chemistry, measuring the rate of the reaction is not simple. A SE-HPLC method was developed to monitor the reaction by recording the loss of protein monomer as the reaction proceeded.

Fig. 2 shows the percentage of monomeric RNAse A remaining in solution, as calculated from the area under the corresponding peak in the chromatogram, as a function of incubation time. The rate of loss of protein monomer can be seen to increase in a consistent fashion with increasing temperature. Thus the reaction may well be significant during the processing of foods, such as grain foods, which contain DHA and are processed at temperatures considerably in excess of those used for these model studies.

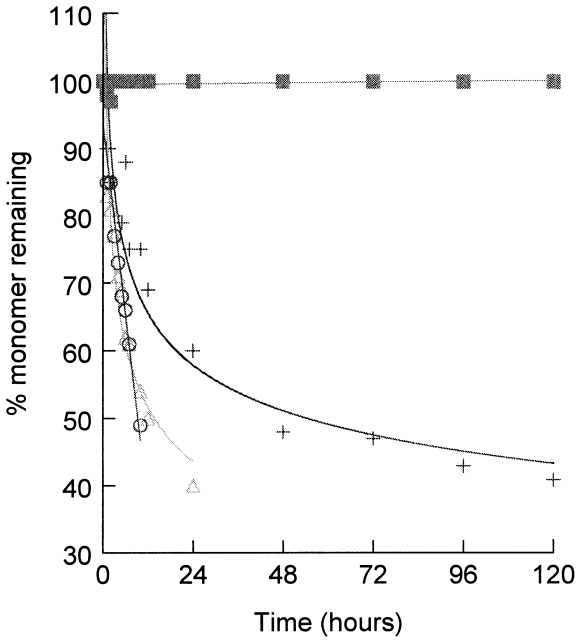

Fig. 2. Monomeric RNAse A content of samples incubated at 37°C (■) 50°C (+), 75°C (▲) and 90°C (●) with a line of best fit.

3.3. Relative rates of the crosslinking reaction mediated by DHA degradation products

DHA is not a stable molecule and degrades to a complex mixture of products at high temperatures (Vernin et al., 1998). We therefore postulated that the crosslinking that we observed may be due to the presence of reactive degradation products of DHA. A number of degradation products of DHA have been identified (Vernin et al.), including 2,3-diketogulonic acid (Slight, Feather & Ortwerth, 1990), threose (Lopez & Feather, 1992) and oxalic acid (Ortwerth, Speaker, Prabhakaram, Lopez, Li & Feather, 1994). Others such as glyoxal (Schwarzenbolz, Henle, Haebner & Klostermeyer, 1997), methyl glyoxal (Larisch, Pischetrider & Severin, 1997) and diacetyl (Ledl & Schleicher, 1990) can be postulated as degradation products of DHA. The relative importance of the breakdown products of DHA in the crosslinking reaction of food proteins has not yet been established. Although this reaction has attracted some attention in the medical arena (e.g. Frye, Degenhardt, Thorpe & Baynes, 1999; Ortwerth et al., 1994; Slight et al., 1990) the crosslinking of food proteins, by DHA and its degradation products, has been somewhat

neglected (Larisch et al., 1996; Nishimura, Ohtsura & Nigota 1989a,b).

In order to gain insights into which of these degradation products might be involved in the crosslinking reaction, several known degradation products of DHA were incubated with RNAse A and their crosslinking ability was analysed by SDS-PAGE. We have previously reported the crosslinking ability of cyclotene (Gerrard, Fayle & Sutton, 1999), a known degradation product of DHA (Velisek, Davidek, Kubelka, Zeliakova & Pokorny, 1976; Vernin et al., 1998). However, since the rate of crosslinking by cyclotene was slower than that by DHA it cannot be responsible for the effects observed in the latter case. Our attention turned, therefore, to other, potentially more reactive, breakdown products of DHA.

In order to study the initial stages of the reaction and get a clear idea of the relative rates of the reaction of RNAse with different molecules, all incubations were carried out at 37°C, where the reactions were readily monitored. Fig. 3 depicts the DHA-mediated formation of crosslinked protein, with increasing time. These crosslinks were not broken by treatment with reducing agents, such as 2-mercaptoethanol. Large protein aggregates were found to form as early as day two of the reaction. By day eight, large aggregates that did not enter the polyacrylamide gel were produced. When all of the amino groups were selectively protected, with succinic anhydride (Hollecker & Creighton, 1980), and the protected protein incubated with DHA as before, no crosslinking was observed, as had previously been observed for cyclotene (Gerrard, Fayle & Sutton, 1999). This provides strong evidence that the crosslinking reaction that we are observing involves a lysine residue in a Maillard reaction.

The reaction of oxalic acid with RNAse A did not result in the formation of protein crosslinks. This is entirely consistent with our hypothesis that the crosslinks observed involve reaction of a carbonyl group with a lysine residue; carboxylic acid groups would not be expected to undergo such a reaction.

Threose, glyoxal and diacetyl were incubated with RNAse A, and all were found to have comparable reactivity. The results for threose are shown in Fig. 4. All three molecules were more reactive than DHA. Thus if DHA degraded to threose, glyoxal or diacetyl in the reaction mixture, these molecules would react preferentially with the protein to form covalent crosslinks. Once again, reaction was not seen if the lysines were protected, providing strong evidence that the reaction involves a carbonyl group and a lysine residue.

Fig. 5 shows the products of the reaction of methyl glyoxal with RNAse A. Crosslinking is very rapid, occurring within the first hour of the reaction, and produces clear evidence of distinct pentameric and hexameric protein during the first day. Beyond this time, aggregation occurs to such an extent that the proteins no longer enter the gel. Thus, any methyl glyoxal produced from the degradation of DHA will be a very reactive crosslinking agent. When the lysines were protected, the reaction was severely inhibited, but some evidence of dimer formation was still evident. This either suggests that other amino acids, such as arginine, may also be involved, or that the methyl glyoxal was able to access lysine residues that the protecting succinic anhydride could not. Further work is underway to distinguish between these two possibilities.

When the polyacrylamide gel of DHA with RNAse A is compared with those of the degradation products, it can be seen that many more products are formed in the reaction of RNAse A with DHA directly. The electrophoretic gels of the reactions containing degradation products contain distinct bands, corresponding to multimeric protein formation. Those involving DHA, however, consistently contain bands that appear smeared. This may be due to the formation of protein

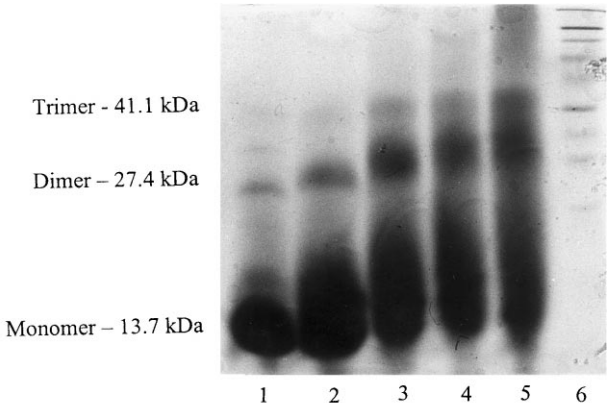

Fig. 3. SDS-PAGE gel showing incubation of RNAse A with DHA. Samples were incubated for up to 8 days at 37°C. Lanes (from left to right): 1, Incubated RNAse A control; 2–5, RNAse A incubated with DHA for 2, 4, 6, and 8 days, respectively; 6. markers.

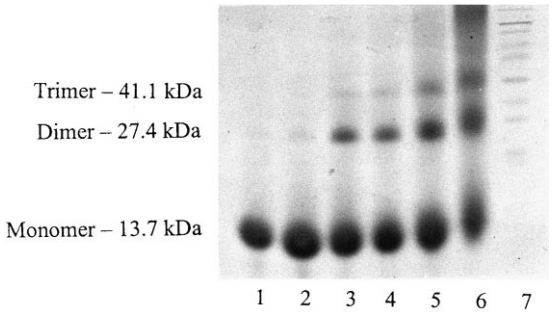

Fig. 4. SDS-PAGE gel showing incubation of RNAse A with threose. Samples were incubated for up to 6 days at 37°C. Lanes (from left to right): 1, fresh RNAse A control; 2, incubated RNAse A control; 3–6, RNAse A incubated with threose for 2 h, 10 h, 2 days, 6 days, respectively; 7. markers.

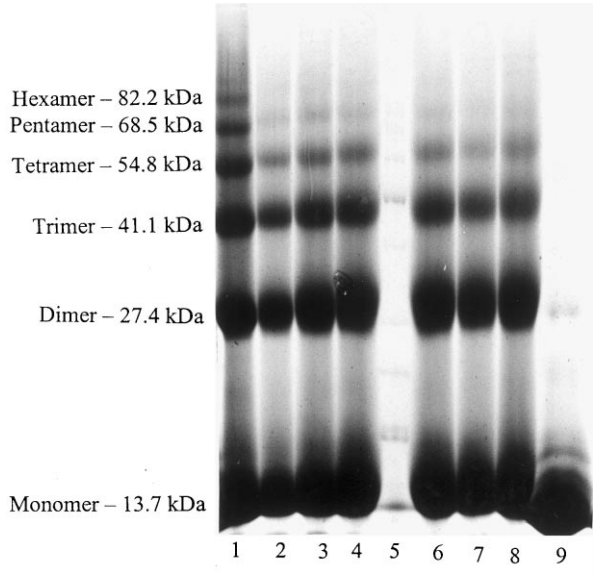

Fig. 5. SDS-PAGE gel showing incubation of RNAse A with methyl glyoxal. Samples were incubated for up to 7 days at 37°C. Lanes (from left to right): 1–4, RNAse A incubated with methyl glyoxal for 7 days, 6 days, 5 and 4 days, respectively; 5, markers; 6–8, 3, 2 and 1 day respectively; 9, incubated RNAse A control.

subunits, via redox chemistry, which are subsequently crosslinked, resulting in products of many different sizes.

The order of crosslinking reactivity was, therefore, found to be methyl glyoxal > glyoxal, threose, diacetyl > DHA > cyclotene > oxalic acid; hence, the presence of the first four degradation products will result in an increased rate of crosslinking. The reactivity of methyl glyoxal has been reported in the medical arena, as a reactive breakdown product of glucose (Frye et al., 1999). The reason for the particular reactivity of methyl glyoxal is not clear, but may be related to the differential reactivity of the two functional groups. Further work is underway to investigate the molecular mechanism of this reaction in detail and explain these observations.

In an attempt to estimate the likely presence of these alternative crosslinking agents in the DHA-RNAse A reaction mixture, a solution of DHA was incubated at 37°C and monitored by ^{1}H and ^{13}C NMR. After 8 days, the NMR spectrum revealed a complex mixture of products, including some remaining DHA. The spectrum was not inconsistent with the presence of the breakdown products above, but what remained was evidently a very complex mixture and not easy to interpret. These results need to be interpreted with caution as the stability of DHA alone does not necessarily correspond with that in the presence of protein. However, it does give some evidence that DHA could still be present in the protein incubations after this length of time.

4. Conclusion

The rate of the Maillard reaction between DHA and the model protein RNAse A was found to increase with increasing temperature. The rate is sufficiently high that this chemistry could be significant during food processing, and may affect the functional properties of the food proteins involved.

Oxalic acid, on the other hand, did not lead to protein crosslinking. Other DHA degradation products — methyl glyoxal, glyoxal, diacetyl and threose — were found to cause protein crosslinking at a greater rate than DHA. All reactions were inhibited by protection of lysine residues, suggesting that the protein crosslinking proceeds via a Maillard reaction between a free lysine residue and a carbonyl compound. The presence of these effective protein crosslinking agents in the DHA-RNAse A reaction mixture would, therefore, result in an increase in the rate of crosslinking. Further work must be carried out to establish which of these reactions, if any, predominate in the reaction of DHA with proteins, which amino acids are involved, other than lysine, and how these reactions affect the functional properties of food proteins.

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