Covalent Protein Adduct Formation and Protein Cross-Linking Resulting from the Maillard Reaction between Cyclotene and a Model Food Protein

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Covalent Maillard products of the reactions of carbonyl compounds with proteins are often described in the literature, but, until recently, evidence for their existence has been indirect. Cyclotene (2-hydroxy-3-methylcyclopent-2-enone), a common flavor compound, was incubated with a model food protein, ribonuclease, and found to cross-link the protein. Size exclusion high-performance liquid chromatrography and electrospray mass spectrometry of the early stages of the reaction provide strong evidence for covalent adducts that we believe to be intermediates in the cross-linking reaction.

Keywords: *Cyclotene; cross-link; Maillard reaction; ribonuclease*

INTRODUCTION

The Maillard reaction is a general term used to describe a complex series of reactions between reactive carbonyl groups, such as those of reducing sugars, and free amine groups, such as those found on proteins (Maillard, 1912). Due to the complexities inherent in studying proteins, particularly in food systems, direct chemical studies of the Maillard reaction generally rely on the use of model systems comprising a sugar and an amino acid [e.g., Ames and Apriyantono (1996)] or a small peptide (Prabhakaram et al., 1996; Sakurai et al., 1990). Despite over 80 years of research in this area, the molecular mechanisms remain far from understood, although an increasing number of Maillard reaction products have been identified in recent years (Ledl and Schleicher, 1990).

Studies on the Maillard reaction of food proteins themselves generally employ indirect methods to provide evidence of Maillard chemistry; these include monitoring the loss of lysine during reaction, browning reactions (Friedman, 1996), or immunochemical detection (Pischetsrieder et al., 1997). The direct study of Maillard products of food proteins has received little attention (Kim et al., 1997) despite excellent recent work in the medical arena (Lapolla et al., 1996; Tang et al., 1996; Traldi et al., 1996).

Cyclotene (2-hydroxy-3-methylcyclopent-2-enone) is a volatile flavor compound commonly encountered in foods, both as a natural product, for example, in coffee beans (Gianturco et al., 1963), and as a synthetic food additive. It is known to play a role in the Maillard reaction (Nishimura et al., 1980) and has been identified as a degradation product of ascorbic acid (Vernin et al., 1998). Unlike many carbonyl compounds known to partake in Maillard chemistry, cyclotene is readily available and has a simple, stable structure that is not prone to redox chemistry. It was, therefore, selected during our studies of the Maillard reaction of proteins.

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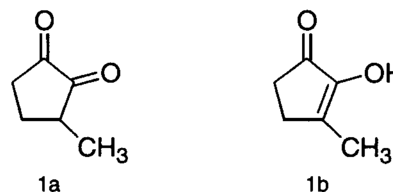

Figure 1. Two of the many possible tautomeric forms of cyclotene.

Cyclotene is generally represented in either of two forms (Figure 1): the dicarbonyl form (**1a**) or the enol form (**1b**). In the crystalline state it has been shown to exist in the enol form (**1b**), but in solution it displays the chemistry of both forms (Fayle et al., 1998).

We report here evidence that cyclotene acts as a protein cross-linking agent and postulate mechanisms for this cross-linking reaction based on direct evidence of Schiff base protein adducts, observed by electrospray mass spectrometry.

MATERIALS AND METHODS

Reagents. Unless otherwise stated, all materials were obtained from Sigma Chemical Co. Cyclotene was obtained as a kind gift from Dr. D. K. Weerasinghe, Firmenich Inc. Ribonuclease A (RNase A) was type XII-A (from bovine pancreas).

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. Visualization of proteins was achieved using Coomassie Brilliant Blue.

Incubations. Unless otherwise stated, incubations were carried out in aqueous solution, at 37 °C, at a protein concentration of 25 mg/mL and a cyclotene concentration of 25 mg/mL. A 15 mg sample of protein was dissolved in 600 μL 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 400 μL of protein solution was transferred to an Eppendorf tube containing 10 mg of cyclotene. After thorough mixing, the solution was equally divided

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into four tubes and placed in an incubator. A tube was removed on alternate days, for 8 days, and stored at −10 °C prior to analysis.

Preparation of RNase with Protected Lysine Residues (Hollecker and Creighton, 1980). RNase A (5 mg) was dissolved in water (3 mL), and the pH was adjusted to 7.0 by dropwise addition of 1 M sodium hydroxide. Succinic anhydride (5 mg) was added gradually to the stirred protein solution over a period of 4 h, with continued monitoring of the pH, and subsequent addition of sodium hydroxide to maintain the pH at 7 (~120 μL). The reaction was judged complete when the pH had remained stable for >10 min and analyzed by urea−PAGE, according to the method of Hollecker and Creighton (1980).

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 sample 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 190−600 nm. The instruments were controlled and data recorded using Waters Millennium 2010 software (version 2.15) operating on a personal computer.

Electrospray Mass Spectrometry. Incubated samples were diluted 1:10 with 50% (v/v) acetonitrile/water prior to injection. Analyses were performed by Wendy Jackson, Technical Officer, Waikato University, New Zealand. Spectra were recorded in positive ion mode on a VG platform II mass spectrometer employing a quadrupole mass filter with an *m/z* range of 0−3000. Injection was via a Rheodyne injector fitted with a 10 μL sample loop. A Thermo Separation Products SpectraSystem P1000 LC pump delivered the solution to the mass spectrometer source (60 °C). Nitrogen was employed both as a drying and as nebulizing gas. Sample components were identified by manual peak picking, using the Transform data processing method included with Masslynx 2.0 software.

RESULTS AND DISCUSSION

Selection of a Model Protein System. In earlier work studying the cross-linking reactions of dehydroascorbic acid (Fayle, 1998; Gerrard et al., 1998), we investigated several proteins for cross-linking studies. RNase gave the best results, which we attribute to its comparatively small size (13.7 kDa) and thermal and chemical stability. RNase was therefore chosen for this study. It contains 11 free amino groups, corresponding to 10 lysine residues and a terminal amino group, all of which are located on the surface of protein (Boqui et al., 1994) and presumably available for reaction. 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 et al., 1994).

Cross-Linking Reactions with Cyclotene. Adapting methodology that we have developed for the study of DHA-mediated protein cross-linking (Gerrard et al., 1998), we incubated ribonuclease in a solution of cyclotene over several days at 37 °C. The subsequent reaction mixtures were analyzed using standard SE-HPLC and PAGE methods. The conditions were chosen to study the initial stages of the Maillard reaction, because the protein aggregates formed in later stages of the reaction, at higher temperatures, have proved too difficult to study thus far.

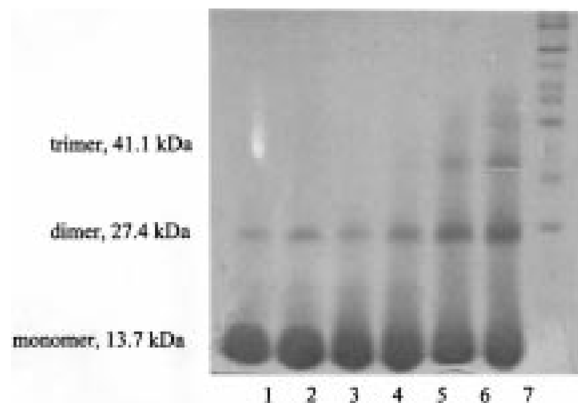

Figure 2. PAGE gel showing incubation of RNase with cyclotene. Samples were incubated for up to 8 days at 37 °C. Lanes (from left to right): 1, fresh RNase control; 2, incubated RNase control (8 days); 3−6, RNase incubated with cyclotene for 2, 4, 6, and 8 days; 7, markers.

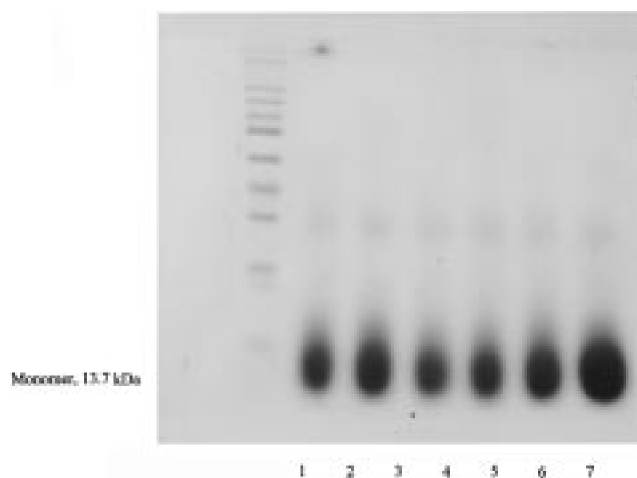

Figure 3. PAGE gel showing incubation of RNase with lysines protected with cyclotene. Samples were incubated for up to 8 days at 37 °C. Lanes (from left to right): 1, markers; 2, fresh RNase control; 3, incubated RNase control (8 days); 4−7, RNase incubated with cyclotene for 2, 4, 6, and 8 days.

Figure 2 clearly demonstrates, for the first time, that cyclotene effects cross-linking of ribonuclease under the conditions of the reaction. The incubated control RNase sample was incubated under conditions identical to those used for samples reacted with cyclotene, but cyclotene was omitted from the tube; analysis by PAGE showed this sample to be identical to the nonincubated control RNase (cf. lanes 1 and 2 of Figure 2), demonstrating that the cross-linking reaction is cyclotene dependent. The reaction was far more specific than the equivalent reaction with DHA, leading to very clean dimer and trimer formation. We assume that in the DHA system, additional redox chemistry is taking place, leading to a range of products and "smeared" polyacrylamide gels (Nishimura et al., 1989; Gerrard et al., 1998); this does not appear to occur in the cyclotene system.

When the reaction was repeated using a sample of ribonuclease in which all of the lysine residues had been protected (Hollecker and Creighton, 1980), no cross-linking reaction was observed. When analyzed by PAGE, the incubated samples were identical to the unreacted control (Figure 3). This provided strong evidence that the cross-linking reaction requires free lysine residues, so we set out to establish the nature of the initial reaction product.

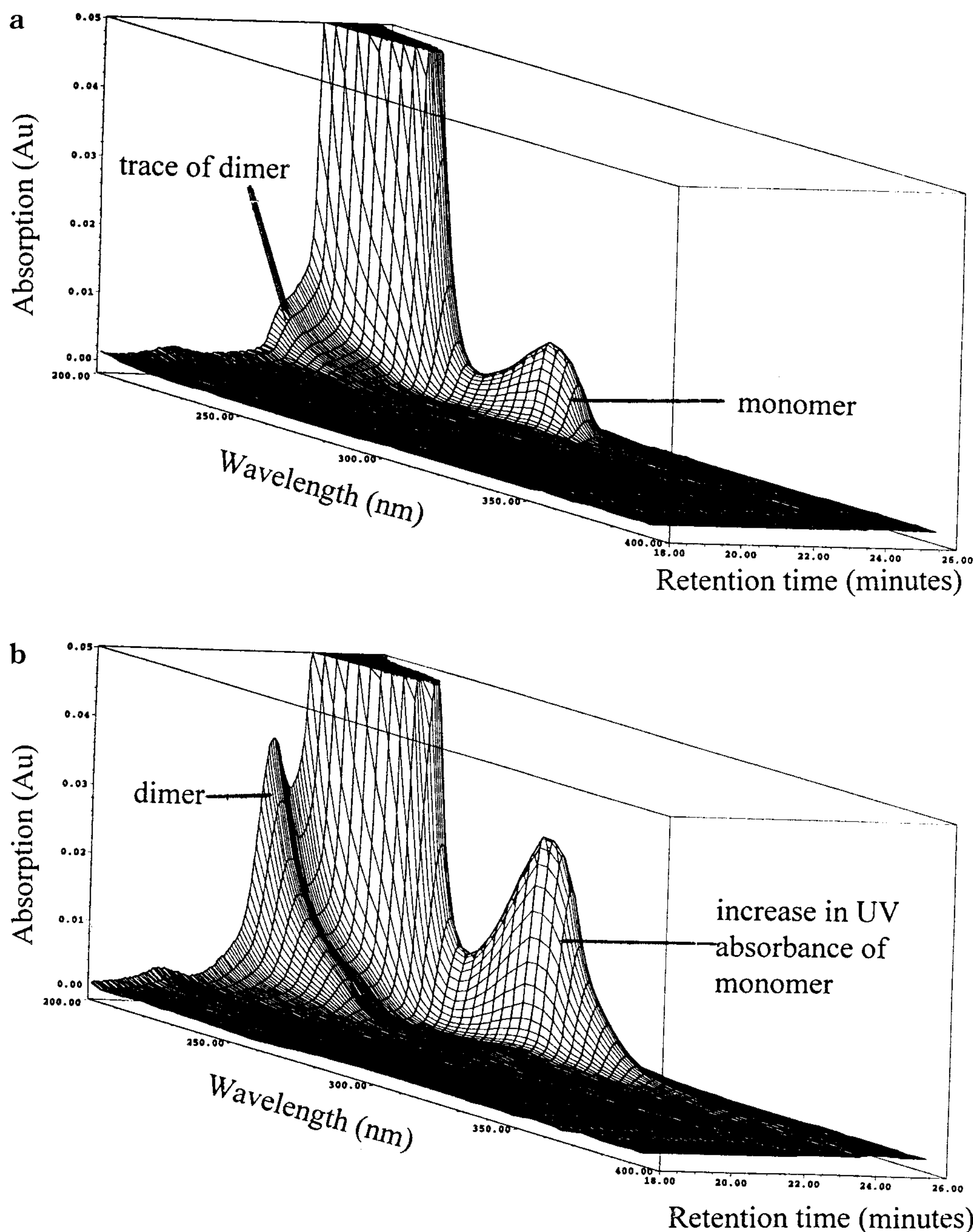

Figure 4. (a) SE-HPLC trace of incubated control RNase; (b) SE-HPLC trace of RNase incubated with cyclotene for 6 days.

Identification of the Intermediate in the Cross-Linking Reaction. Analysis by SE-HPLC with diode array detection allowed us to study the UV−visible absorption spectrum of the monomeric protein as the reaction progressed. Figure 4 shows a comparison of a control solution of unreacted ribonuclease (Figure 4a) and a sample that had been incubated with cyclotene for 6 days (Figure 4b). In addition to the formation of the dimer at a retention time of 23 min, a clear shift in the UV−visible absorption spectrum was observed for the monomeric protein, at a retention time of 25 min. As the reaction proceeded, the absorbance of the protein increased considerably between 280 and 350 nm (absorption maximum = 310 nm). This is consistent with formation of a Schiff base adduct between a lysine residue of ribonuclease and a cyclotene molecule. The shift in the absorption envelope was observed only if the samples were analyzed directly after removal from the reaction mixture.

Samples that had been stored prior to analysis showed the same degree of dimer formation as the freshly analyzed samples, but the UV absorbance of the monomer peak was similar to that of the controls. This is also consistent with Schiff base formation, because such an adduct would presumably be labile in aqueous solution. The dimeric species did not show increased absorption in the 280−350 nm region. This is consistent with the Schiff base of the monomer reacting with a further monomer to form the dimer and losing the absorption that corresponded to the Schiff base.

To obtain further information on the identity of the protein adduct, we carried out electrospray mass spectrometry on the incubated mixture of cyclotene and ribonuclease. Figure 5a shows the electrospray mass spectrum of the control sample of ribonuclease. A single series (A) of peaks is observed, suggesting a molecular ion of mass of 13681 ± 5.37 Da, consistent with the reported mass of 13680 Da (Julien et al., 1994). Figure

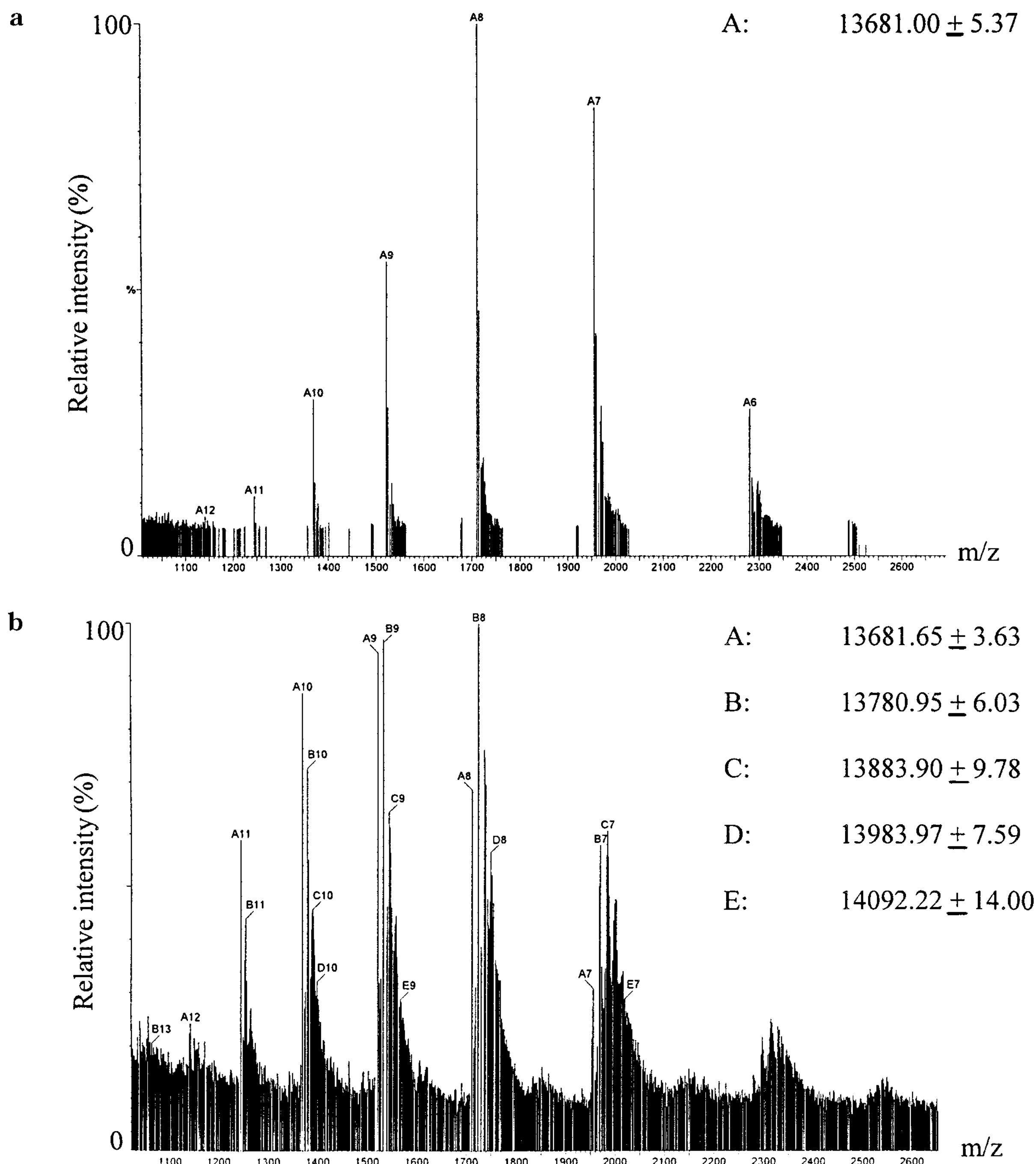

Figure 5. (a) Electrospray mass spectrum of incubated control RNase; (b) electrospray mass spectrum of RNase incubated with cyclotene for 6 days.

5b shows the electrospray mass spectrum of the reaction products formed from ribonuclease and cyclotene. Here, five series of peaks were visible. The first, series A, corresponds to unreacted protein, as seen in the control. The second, series B, corresponds to the addition of one molecule of cyclotene and subsequent dehydration. We propose that this is the Schiff base intermediate that was visible in the SE-HPLC trace (Figure 4b, above). We further propose that this Schiff base is the key intermediate in the cross-link formation with cyclotene. This is illustrated in Figure 6. Work is currently underway to establish the next step of the reaction and to identify the chemical nature of the covalent cross-link in the dimeric protein.

An alternative reaction with a free arginine residue of ribonuclease might have been predicted from the known chemistry of dicarbonyl compounds and proteins (Riordan, 1979). However, such a reaction is inconsistent with our observed masses (Figure 7).

Three other observed series of peaks in the mass spectrum (Figure 5b), C, D, and E, presumably correspond to the addition of further molecules of cyclotene to the Schiff base adduct. The masses are not consistent with the formation of a simple series of Schiff bases, as shown in Table 1. We suggest that the second and fourth molecules of cyclotene add without dehydration. The molecular details of this process are not yet clear, but further studies are underway to establish them.

The combined use of SE-HPLC and electrospray mass spectrometry gives key insights into the Maillard reaction of intact proteins, of direct relevance to food systems. Current work is focusing on subsequent stages

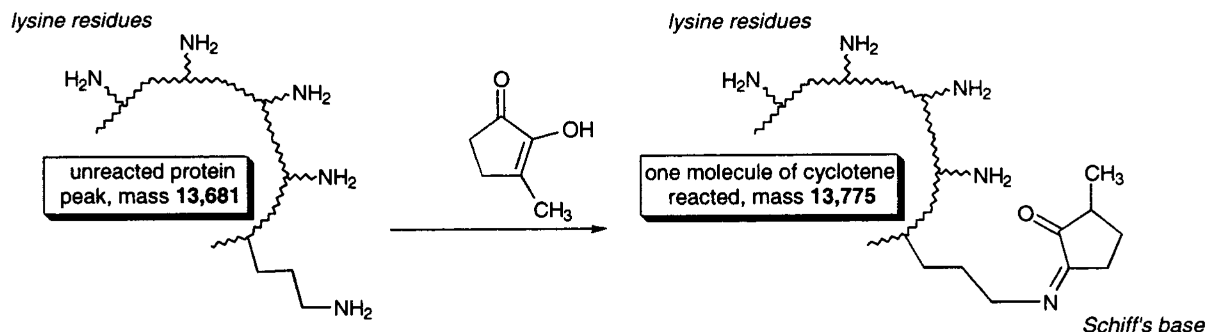

Figure 6. Proposed formation of Schiff base intermediate.

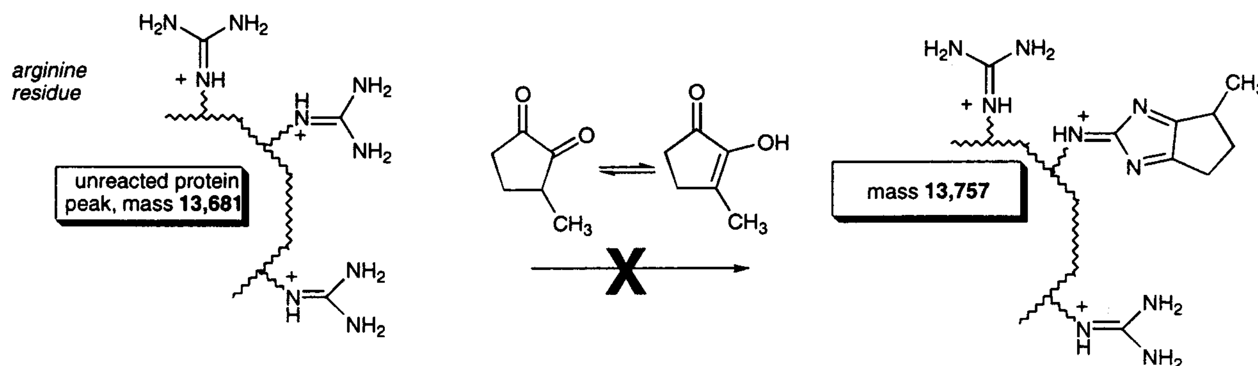

Figure 7. Discounted alternative possibility of reaction at arginine.

Table 1

no. of cyclotene molecules reacted	calcd mass of obsd protein adduct (A), assuming each forms a Schiff base	calcd mass of obsd monohydrate (A + 18)	calcd mass of obsd dihydrate (A + 36)	recorded mass
0	**13681**	13699	13717	13681.65 ± 3.63
1	**13775**	13793	13811	13780.95 ± 6.03
2	13869	**13887**	13905	13883.90 ± 9.78
3	13963	**13981**	13999	13983.97 ± 7.59
4	14057	14075	**14093**	14092.22 ± 14.0

of the reactions, specifically in characterization of the dimeric and aggregated species. We are also examining whether the same reactions occur under conditions met during high-temperature food processing.

ABBREVIATIONS USED

RNase, ribonuclease A; PAGE, polyacrylamide gel electrophoresis; SE-HPLC, size exclusion high-performance liquid chromatography.

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