Mechanisms of protein modification during model anti-viral heat-treatment bioprocessing of β-lactoglobulin variant A in the presence of sucrose

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To ensure the safety of plasma and recombinant therapeutic proteins, heat treatment is routinely applied to these biopharmaceuticals as a means of virus inactivation. However, to maintain protein integrity during heat treatment it is necessary to use high concentrations of thermostabilizing excipients, such as sucrose, in order to prevent protein damage. In this study we describe the covalent modifications inferred to a model protein, β-lactoglobulin A, that occur during typical and extended anti-viral heat treatments. The chemical derivation and mechanisms by which these modifications arise are addressed. Heat treatment initiated hydrolysis of sucrose to glucose and fructose, which in turn were degraded to glyoxal. Glyoxal and the free reducing sugars reacted with free amino groups in β-lactoglobulin A to yield Maillard glycation adducts and advanced glycation end products (AGEs). The major mechanism for AGE formation was via degradation of glucose-derived Schiff-base adducts. Heat treatment and glycation of β-lactoglobulin A resulted in thiol-disulphide interchange reactions leading to protein oligomerization. A small population of β-lactoglobulin A non-disulphide-linked dimers were also observed with increasingly harsh heat treatments. These findings have implications for (i) improvements in the safety and efficacy of heat-treated protein biopharmaceuticals and (ii) our understanding of the mechanisms of protein glycation and AGE adduct formation.

Introduction

The viral inactivation of therapeutic protein products derived from both plasma and recombinant sources has received considerable interest since the outbreak of HIV infection in haemophiliac patients during the early 1980s. Although currently favoured viral-inactivation methods include heating the protein in solution or freeze-dried state, solvent detergent treatment, nanofiltration and UV irradiation, heat treatment is the current 'gold standard' against which other methods are judged, and many therapeutic protein products are treated in this way (e.g. albumin, Factor VIII, Factor IX). Furthermore, severe heat treatment is one of the few methods that can be applied to the finished product in its container. However, as proteins rapidly denature in heated solution, lyophilization and the use of thermostabilizing excipients such as polyhydric alcohols, sugars and amino acids have been utilized to promote thermostability during heat treatments. Such agents preferentially hydrate proteins by being themselves excluded from the vicinity of the protein and effectively reduce protein concentration, therefore minimizing the rate at which damaging intermolecular pathways, such as aggregation, proceed at high levels of hydration. In the case of Factor VIII, which is used to treat haemophilia A, large amounts of sucrose and glycine are added to protein preparations prior to heat treatment in the liquid state at 60 °C for 10 h [1]. While this heat treatment eliminates most viruses, chemical modification of the protein can also result, leading to the formation of neoantigens [2–4]. Subsequently some patients have produced neutralizing antibodies that render them refractory to further treatment [5]. Furthermore, Knevelman et al. [6] have reported that the hydrolysis of sucrose to fructose and glucose during autoclaving of freeze-drying buffers can have a detrimental effect on the activity of Factor VIII samples after subsequent dry heat treatment.

We have shown recently that model anti-viral heat treatments of lysozyme samples formulated in sucrose and glycine leads to the covalent modification of lysozyme [7]. These covalent modifications were shown unequivocally to be due to Maillard-reaction adducts, whereby glucose and fructose react with the free amino groups of lysine residues on the surface of the protein. This non-enzymic reaction is

Key words: advanced glycation end product, protein glycation, thiol–disulphide cross-linking.

Abbreviations used: AGE, advanced glycation end product; β-LGa, bovine β-lactoglobulin variant A; CML, N^{ε}-carboxymethyl-lysine; ESI-MS, electrospray ionization MS; LC-ESI-MS, liquid chromatography ESI-MS; DTPAA, diethylenetriaminepenta-acetic acid.

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possible due to the hydrolysis of sucrose to yield glucose and fructose during anti-viral heat-treatment bioprocessing. The Maillard reaction proceeds via the initial formation of a Schiff-base adduct between the reducing sugar and the amino group, followed by a rearrangement to a more stable Amadori product in the case of glucose [8] or Heyns product in the case of fructose [9]. The Amadori and Heyns products can then, over a period of months or years, undergo a further series of poorly understood reactions to yield numerous modified protein adducts known collectively as advanced glycation end products (AGEs) [10]. AGEs may be formed *in vivo* on a variety of blood proteins and are implicated in the pathology of disease such as diabetes and Alzheimer's [11]. In addition to our earlier study on lysozyme, Schalkwijk et al. [12] have recently reported the presence of AGE products in glucose-based peritoneal dialysis fluids after anti-viral heat treatment. In these peritoneal fluids the majority of *in vitro* AGE products observed after heat sterilization were formed from the reaction of proteins with 1,2-dicarbonyl compounds derived from glucose autoxidation during anti-viral heat treatment [12].

Although we have shown previously that the temperatures required during bioprocessing to achieve the desired virus-inactivation levels in therapeutic protein formulations facilitates sucrose hydrolysis and the formation of Maillard glycation adducts [7], little is currently known about the mechanisms of thermal inactivation of proteins due to glycation during anti-viral heat treatment at elevated temperatures. We therefore undertook this study in order to (i) elucidate the chemical mechanisms of protein modifications and (ii) enhance our current understanding of protein glycation and AGE adduct formation in solution during anti-viral heat treatments. We report here the identity of protein modifications and aggregates that form during anti-viral heat treatments of bovine β-lactoglobulin variant A (β-LGa), the identity of the dicarbonyl sugars formed during heat treatment of sucrose formulations and their role in AGE product formation, and the mechanistic route by which glycation, AGE adduct formation and oligomerization proceed during anti-viral heat treatment of protein samples formulated in sucrose.

Experimental

Materials

Unless otherwise stated, all chemical reagents were of the highest quality available from Sigma (Poole, Dorset, U.K.), including β-LGa (from bovine milk, product L-7880). (Carboxymethyl)trimethylammonium chloride hydrazide (Girard's Reagent T) and glyoxal (40%, w/v, in water) were obtained from Aldrich. Prestained SDS/PAGE broad-range markers were from Bio-Rad Laboratories (Hemel Hempstead, Herts., U.K). HPLC solvents were obtained from BDH Laboratories (Poole, Dorset, U.K.).

Protein formulations and anti-viral heat-treatment conditions

β-LGa samples were formulated at 5 mg·ml^{-1} in a standard bioprocessing formulation: 1.75 M (60% w/v) sucrose, 1.5 M glycine and 10 mM lysine, pH 7. Protein samples were prepared by dissolving β-LGa in 10 mM lysine (pH 7) buffer, followed by the addition of the required amount of sucrose and glycine. Lysine buffer was then used to correct the volume so that the required formulation conditions were achieved. Aliquots (1 ml) were then dispensed into stoppered glass vials, placed in a dry-block set at 60 °C and subjected to heat treatments for 10 h (standard bioprocessing) and extended heat treatments for 72 and 144 h. For samples formulated in the presence of 50 mM glucose or 50 mM fructose, these sugars were added to the solution and the sucrose in the formulation replaced with 60% (w/v) sorbitol. For experiments requiring anti-oxidative conditions, samples were additionally formulated in 1 mM concentrations of the metal-ion chelators phytic acid and diethylenetriaminepenta-acetic acid (DTPAA), after which the solutions were flushed with oxygen-free nitrogen gas before the vials were evacuated and heat treatments undertaken as described above. After heat treatment all samples were stored at 4 °C prior to analysis.

Electrospray ionization MS (ESI-MS)

Mass spectra were recorded in the positive-ion mode on a Finnigan MAT LCQ ion-trap mass spectrometer. The charge-state ion distributions observed in ESI mass spectra were deconvoluted using Finnigan Corps BioExplore software (version 1.0 for LCQ 1.2). Sucrose, glucose, fructose and additional formulation components were removed before protein samples were introduced into the electrospray source by reversed-phase HPLC using a Phenomenex® Jupiter C_4 50 mm × 2 mm column (Phenomenex, Macclesfield, Cheshire, U.K.) linked to a Hewlett–Packard 1100 series HPLC, as previously described [7].

Sucrose hydrolysis

The rate of sucrose hydrolysis during anti-viral heat treatments was calculated by measuring the concentration of glucose in β-LGa samples after heat treatment using a hexokinase-based glucose assay kit (Sigma) as described in the manufacturer's instructions.

HPLC and spectrophotometric analyses of 1,2-dicarbonyls

1,2-Dicarbonyls formed during anti-viral heat treatment of β-LGa samples formulated in 1.75 M sucrose, 50 mM glucose or 50 mM fructose were identified using the Girard-T HPLC assay system described by Wells-Knecht et al. [13]. The dicarbonyl Girard-T adducts formed after heat-treated samples (250 μl) were incubated in 0.5 M sodium formate buffer (pH 2.9, 750 μl) and 50 μl of stock Girard-T reagent (0.5 M) at room temperature for 24 h were purified on 1-ml C_{18} solid-phase Sep-Pak extraction cartridges from Waters in solvents containing 0.25% heptafluorobutyric acid. The purified dicarbonyl Girard-T adducts were dried in a speed-vacuum concentrator, resuspended in 500 μl of water and then 100 μl aliquots analysed on a Phenomenex® Jupiter C_{18} reverse-phase column (250 mm $\times$ 2.0 mm inner diameter) using the heptafluorobutyric acid-containing buffer system and gradient described by Wells-Knecht et al. [13]. The rate of formation of 1,2-dicarbonyls during heat treatment was monitored using the Girard-T spectrophotometric assay at pH 2.9 also described in [13]. Briefly, 100 μl of heat-treated sample was mixed with 900 μl of 0.5 M sodium formate buffer (pH 2.9) and 50 μl of stock Girard-T reagent added. After incubation at room temperature for 30 min the absorbance of samples was read at a wavelength of 294 nm. A standard curve prepared with the 1,2-dicarbonyl glyoxal was used to calculate the concentration of dicarbonyls in heat-treated samples.

Gel electrophoresis

SDS/PAGE of heat-treated samples was undertaken using 10% Bis-Tris NuPAGE gels and NuPAGE Mes buffer from Novex Electrophoresis GmbH (Frankfurt, Germany). Gels were run using the pre-programmed method for NuPAGE gels on a Novex PowerEase500 power pack. For reducing gels, samples were prepared in the presence of 100 mM dithiothreitol. Protein bands were detected with Coomasie Brilliant Blue R250 stain. Image analysis of SDS/PAGE gels was undertaken using the ImageMaster 1D Elite Gel Analysis software package from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.).

Results

Protein formulation and anti-viral heat-treatment bioprocessing

Unheated liquid formulations containing 60% sucrose and heat-treated formulations containing sorbitol were clear and colourless after pasteurization. A correlation between the length of heat treatment and colour of the β-LGa solutions was clearly evident, with longer heat treatments resulting in a more intense yellow colouring. β-LGa samples formulated in 50 mM glucose or 50 mM fructose often contained precipitated material after extreme heat treatment (60 °C, 144 h).

β-LGa is covalently modified during anti-viral heat treatment in the presence of sucrose

β-LGa is a relatively small protein (18363 Da) that is highly amenable to direct analysis by ESI-MS to define covalent modifications [14–16]. β-LGa samples that had been formulated and heat treated at 60 °C in 1.75 M sucrose, 1.5 M glycine and 10 mM lysine could therefore be quickly analysed for covalent modifications by liquid chromatography ESI-MS (LC-ESI-MS). The mass spectra of all unheated but formulated control samples of β-LGa (Figure 1A), and of those samples formulated and heat treated in 60% sorbitol, were almost identical with that of native, unformulated β-LGa, confirming that neither the formulation process nor heat treatment alone were affecting the molecular integrity of the protein. The deconvoluted spectrum resulted in one major peak with a molecular mass of 18364 Da and a minor contaminant peak of 18688 Da (Figure 1A). The higher-molecular-mass contaminant species (324 Da greater than the native mass) can be structurally assigned to protein modification resulting from the condensation of two units of glucose (or fructose) with a primary amino group on the surface of the protein. This non-enzymic reaction has been well characterized and the mass change observed here (162 Da per glucose unit) is consistent with those previously reported for the glycation of proteins in the presence of relatively high concentrations (0.25 M) of glucose and fructose [17–19]. This di-glycated contaminant adduct was always observed in both native and unheated control samples of β-LGa and could not therefore be due to the formulation process.

Direct LC-ESI-MS analysis of β-LGa samples formulated in 60% sucrose, 1.5 M glycine and 10 mM lysine that had been pasteurized at 60 °C showed conclusively that heat treatment in the presence of sucrose resulted in protein modifications (Figures 1B–1D). The LC-ESI-MS spectrum of β-LGa samples formulated in sucrose became more complex as the length of heat treatment increased (Figure 1), so that although the peak corresponding to native, unmodified β-LGa (18364 Da) remained the most abundant in all cases, several other distinct species increased in relative abundance with time. The initial protein-modification observed (after heat treatment for 10 h at 60 °C) was a distinct species present at 18526 Da (Figure 1B), a mass increase of 162 Da above that of native β-LGa, corresponding to the condensation of 1 mol of glucose or fructose (derived from the hydrolysis of sucrose during heat treatment) with the amino group of a lysine residue on the β-LGa molecule. We note that both the glucose glycation product, the Amadori product, and the fructose glycation product, the Heyns

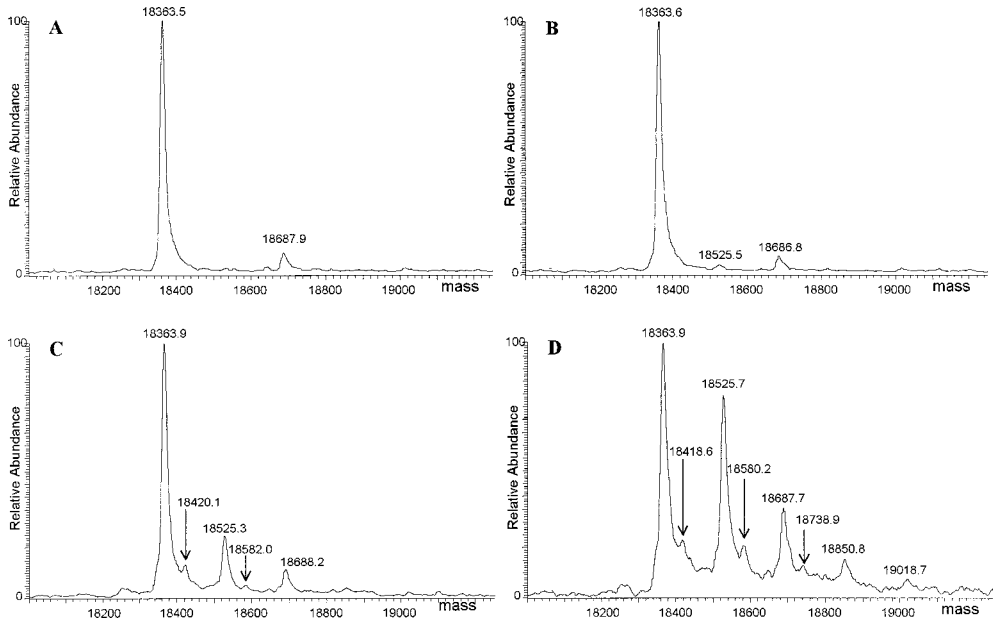


Figure 1 ESI-MS analyses of β-LGa samples heat treated in the presence of sucrose

Samples were formulated (at 5 mg·ml^{-1}) in 1.75 M (60%) sucrose, 1.5 M glycine and 10 mM lysine and subjected to heat treatment at 60 °C for (**A**) 0 h (unheated control), (**B**) 10 h, (**C**) 72 h and (**D**) 144 h.

product, result in a mass increase of 162 Da and hence we collectively refer to these as 'glycation products'. It is not possible to determine from the data presented in Figure 1 whether the glycation products derived from glycation by glucose, fructose or a combination of these reducing sugars. We also note that although previous studies have reported the condensation of glucose and fructose at amino acids other than lysine (such as arginine), initial reaction by these reducing sugars at other amino acids in the present study can be discounted due to the observed mass shift (162 Da) in Figure 1(B). Such a mass shift can only arise from the condensation of sugar molecules at lysine residues and not by reaction at other amino acids. Furthermore, when amino acid analysis was undertaken on samples formulated in 1.75 M sucrose, 1.5 M glycine and 10 mM lysine, pH 7, and heat-treated for 144 h at 60 °C, lysine levels decreased by approx. 2 mol/β-LGa monomer, consistent with modification at lysine residues, while arginine levels remained more or less unchanged.

Increasing the period of anti-viral heat treatment resulted in an increase in the abundance of the monoglycated peak (18526 Da, Figures 1C and 1D) such that after 144 h its relative abundance was approx. 80% of the native, unmodified peak (Figure 1D). More highly modified glycation adducts were also observed with increasing periods of anti-viral heat treatment, including an increase in the abundance of the di-glycated β-LGa species (18688 Da, Figures 1C and 1D), inferring that extra di-glycated material accumulated in addition to that initially observed in the native spectra. Tri-glycated (18851 Da) and tetra-glycated (19019 Da) β-LGa species were also present in samples subjected to extended (144 h) heat treatment (Figure 1D). Thus the total yield of glycation products increased with pasteurization. Furthermore, in β-LGa samples heat treated at 60 °C for 72 h in the presence of 50 mM glucose the mono-glycated (18525 Da), di-glycated (18687 Da) and tri-glycated (18850 Da) adducts were all prevalent (Figure 2A), providing further evidence that the products observed in Figure 1 are β-LGa glycated adducts. However, when experiments were undertaken with 50 mM fructose, of the glycation products described above, only the mono-glycated adduct (18527 Da) was present after heat treatment for 72 h (Figure 2B). Thus under the bioprocess conditions utilized in this study, the presence of glucose in protein formulations due to sucrose hydrolysis is far more damaging in terms of the formation of covalently modified β-LGa glycation adducts than the presence of fructose, which appears to react much more slowly.

When β-LGa samples formulated in 1.75 M sucrose were heat-treated for 72 or 144 h the glycation adducts described above were accompanied by various other species, the most abundant of which were broad peaks centred at values of 18420 and 18582 Da (Figure 1C), although after prolonged heat treatment the mass values shifted slightly (Figure 1D). These broad peaks corresponding to modified β-LGa adducts (18419 and 18578 Da) were also present in samples formulated in 50 mM glucose that had been heat-treated for 72 h (Figure 2A). Surprisingly, other than the mono-glycated product described above, the mass spectrum

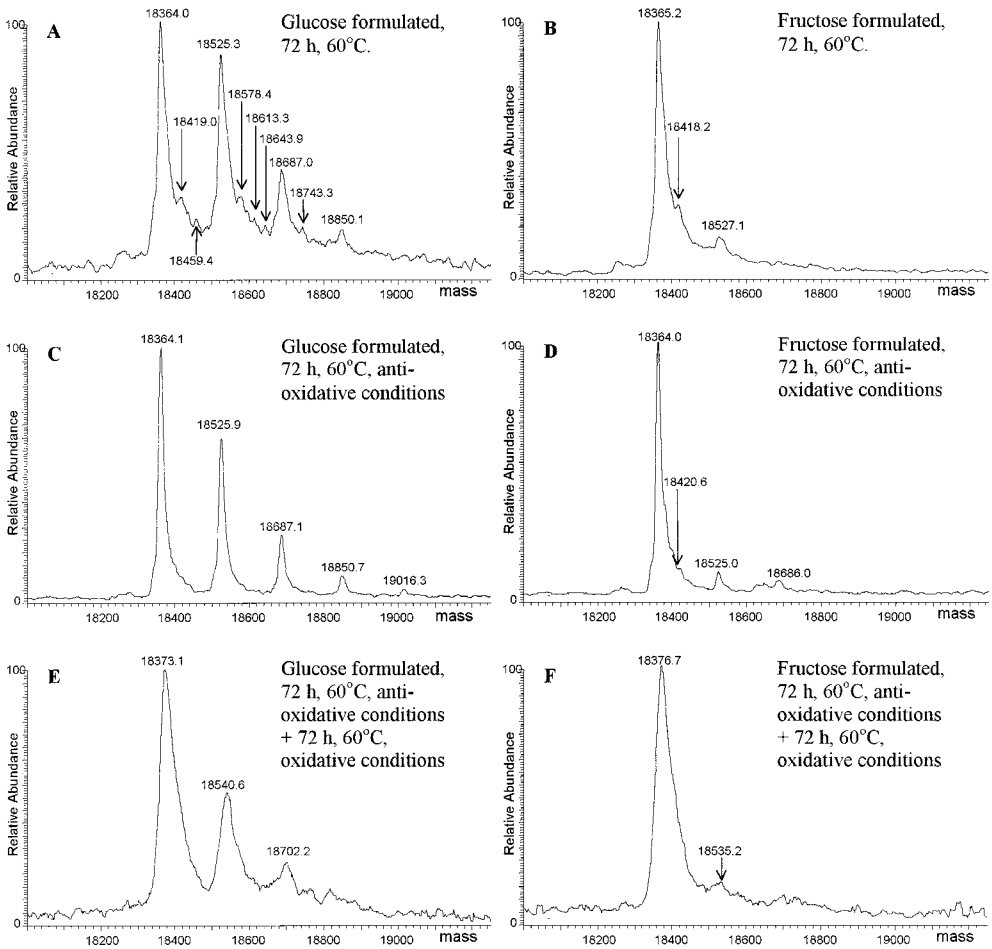


Figure 2 ESI-MS analyses of β-LGa samples heat treated in the presence of glucose and fructose

Samples were formulated (at 5 mg·ml^{-1}) in 50 mM glucose (**A**) or 50 mM fructose (**B**), 60% sorbitol, 1.5 M glycine and 10 mM lysine and subjected to heat treatment at 60 °C for 72 h. (**C** and **D**) Spectra obtained when samples formulated in glucose (**C**) and fructose (**D**) were heat treated under anti-oxidative conditions. When the samples in (**C**) and (**D**) were desalted and reformulated in 60% sorbitol, 1.5 M glycine and 10 mM lysine before heat treatment at 60 °C for a further 72 h under oxidative conditions, the spectra shown in (**E**) (initially in glucose) and (**F**) (initially in fructose) were obtained.

of β-LGa samples formulated and heat-treated in 50 mM fructose for 72 h consisted of just a single modified product centred at 18418 Da (Figure 2B). These additional broad peaks could be due to either the well-documented but poorly understood rearrangement, enolization, fragmentation and oxidation procedures that glycated adducts are known to undergo [8,20,21] or, alternatively, could be derived from reaction of the protein with monosaccharide autoxidation products independent of the glycation adducts. We note that both of these mechanisms could lead to similar or identical protein AGE adducts and that the mass difference associated with these peaks (approx. 56 Da) can be assigned to two-carbon/two-oxygen-containing AGE modifications.

Heat treatment of β-LGa samples formulated in 50 mM glucose or 50 mM fructose in the presence of the transition-metal chelators phytic acid and DTPAA under anaerobic conditions resulted almost exclusively in the formation of β-LGa glycation adducts (162-Da mass differences; Figures 2C and 2D). Under anti-oxidative conditions in the presence of chelators the β-LGa AGE adducts observed in samples pasteurized under oxidative conditions (e.g. 18419 and 18578 Da; Figure 2A) were not observed upon heat treatment. This confirms the requirement for oxygen and traces of transition metals for the formation of the additional β-LGa AGE adducts. We also note that the formation of the dicarbonyl glyoxal from glucose and fructose requires the presence of oxygen and transition-metal ions. When the transition-metal chelators and initial formulation components were removed from samples heated under anti-oxidative conditions, in the samples reformulated under oxidative conditions in 60% sorbitol, 1.5 M glycine and

10 mM lysine and then subjected to heat treatment for a further 72 h the AGE products observed in Figures 2(A) and 2(B) were not reproduced (Figures 2E and 2F).

Formation of reducing sugars and 1,2-dicarbonyl sugars during anti-viral heat treatments

Anti-viral heat treatment of 60% sorbitol, 1.5 M glycine and 10 mM lysine formulations did not result in significant increases in the reducing-sugar content of samples. However, anti-viral heat treatment at 60 °C of a 1.75 M sucrose, 1.5 M glycine and 10 mM lysine, pH 7, formulation resulted in sucrose hydrolysis (as measured by glucose release) to yield glucose and fructose in a linear and constant manner over the 144 h sampling period. The rate of sucrose hydrolysis during heat treatment at 60 °C (measured as glucose release) was 105±5 μmol·l^{-1}·h^{-1} (0.006% of the initial sucrose content·l^{-1}·h^{-1}), in agreement with previous studies [7]. We infer that the total reducing-sugar content is twice the measured glucose content, therefore a sucrose-hydrolysis rate of 105±5 μmol·l^{-1}·h^{-1} yields approx. 30 mM of reducing sugars (glucose and fructose) in 144 h.

The presence of glucose and fructose in protein formulations as a result of sucrose hydrolysis during anti-viral heat treatment at elevated temperatures results in potentially damaging protein modifications, as described above. Furthermore, as glucose itself has been shown to undergo further autoxidation reactions to yield potentially damaging dicarbonyl adducts [13], and these compounds have been shown to be involved in the formation of AGEs [12,22–24], we set out to identify any dicarbonyls present in heat-treated samples. Specifically, we examined the autoxidation of glucose and fructose upon anti-viral heat treatments in the presence of 1.5 M glycine and 10 mM lysine, pH 7. Glucose samples (50 mM glucose, 1.5 M glycine and 10 mM lysine, pH 7) heat-treated at 60 °C for 144 h in the presence of oxygen almost exclusively yielded a single dicarbonyl adduct (as determined by HPLC) which was eluted at the same time as a standard glyoxal sample (Figure 3). The mixing of this sample with a glyoxal standard resulted in co-elution of the samples, both confirming the identification of the dicarbonyl as glyoxal and consistent with the observations of Wells-Knecht et al. [13], who have previously reported that glyoxal is the only dicarbonyl autoxidation product formed from glucose. However, as far as we are aware there has been no study on the dicarbonyl products formed upon autoxidation of fructose. Analysis of fructose samples showed that, as in the case of glucose, upon anti-viral heat-treatment autoxidation yielded glyoxal as the major dicarbonyl product (Figure 3). It was not therefore surprising that analysis of sucrose heat-treated samples revealed that glyoxal was the only dicarbonyl sugar detected in significant amounts (Figure 3). In all cases (sucrose, glucose and fructose) small amounts of various other peaks were

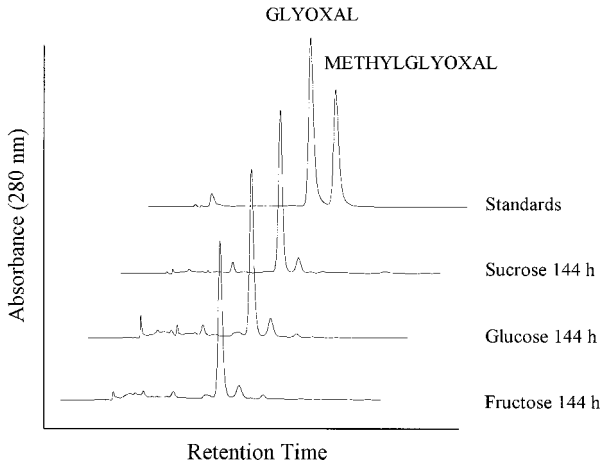


Figure 3 Glyoxal is the dicarbonyl sugar formed during autoxidation of glucose and fructose during anti-viral heat treatment

Shown are the HPLC chromatograms of the Girard-T adducts of a standard mixture of dicarbonyls (glyoxal and methylglyoxal), and the dicarbonyl sugars formed when β-LGa samples were formulated in 1.75 M sucrose, 50 mM glucose or 50 mM fructose and heat-treated at 60 °C for 144 h.

detected in the reversed-phase HPLC chromatograms of heat-treated samples (Figure 3), presumably due to the presence of trace amounts of other dicarbonyls. Dicarbonyls were not observed when samples were additionally formulated with 1 mM DTPAA and 1 mM phytic acid, flushed with oxygen-free nitrogen gas and evacuated before heat treatment, confirming the need for oxygen and trace metals to form dicarbonyl sugars from glucose and fructose.

The Girard-T reagent assay was initially developed for the measurement of glyoxal and was therefore ideal for measuring the rate of glyoxal formation upon heat treatment of sucrose, glucose and fructose formulations. When the standard sucrose-containing bioprocessing formulation was heat-treated at 60 °C the concentration of glyoxal was found to increase in a linear and constant manner at a rate of 0.154±0.015 μmol·l^{-1}·h^{-1} (0.073% of the rate at which glucose and fructose are generated) over the 144 h sampling period. Glyoxal also accumulated at a linear and constant manner over the 144 h sampling period when 50 mM solutions of glucose and fructose were heat-treated at rates of 0.716±0.013 and 0.806±0.023 μmol·l^{-1}·h^{-1}, respectively. Therefore, we assume that glucose and fructose contribute approximately equally to the levels of glyoxal measured in sucrose heat-treated samples over the sampling period. However, heat treatment of a solution consisting of 90 μM glyoxal, 1.5 M glycine and 10 mM lysine, pH 7, at 60 °C showed that glyoxal 'degradation' occurred rapidly in an exponential-decay fashion such that after 48 h almost all of the initial glyoxal was depleted. When glycine was removed from the formulation the rate of glyoxal degradation

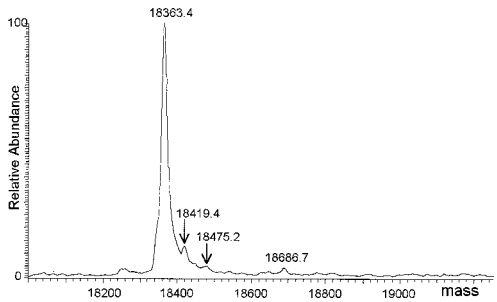


Figure 4 ESI-MS analyses of β-LGa heat-treated at 60 °C for 72 h in the presence of glyoxal

The sample was formulated at 5 mg·ml^{-1} in 60% sorbitol, 100 μM glyoxal, 1.5 M glycine and 10 mM lysine.

diminished significantly so that more than 80% of the initial glyoxal was still present after 48 h of heat treatment.

The LC-ESI-MS spectrum of a β-LGa sample heat-treated in the presence of 100 μM glyoxal at 60 °C for 72 h is shown in Figure 4. The spectrum consisted of one major peak corresponding to native, unmodified β-LGa (18363 Da) and a series of much smaller modified β-LGa peaks, including the di-glycated contaminant adduct present in the starting material (18687 Da). The most prominent of the higher-molecular-mass β-LGa adducts was a broad peak centred at 18419 Da (Figure 4), corresponding to the major non-Amadori or non-Heyns adduct observed in sucrose-, glucose- and fructose-formulated and heat-treated samples (Figures 1 and 2). Once again the mass shift (56 Da) between this peak and the native protein can be assigned to 2-carbon/2-oxygen-containing AGE modifications. Although the structure and composition of the majority of AGE adducts have yet to be established, this mass shift (56 Da) fits that expected for the known reaction of glyoxal with the amino group of a lysine residue to yield the AGE product *N*-carboxymethyl-lysine (CML) [13]. We therefore tentatively assign the modified β-LGa products observed at 18419, 18580 and 18739 Da in sucrose, glucose and fructose heat-treated samples as a CML-modified adduct, a mono-glycated, CML-modified adduct and a di-glycated, CML-modified adduct respectively. A second glyoxal adduct at a further mass shift of 56 Da (18475 Da) was also present in the deconvoluted mass spectrum of β-LGa samples heat-treated in the presence of 100 μM glyoxal (Figure 4), indicative of a di-CML β-LGa adduct.

Aggregate analysis

All β-LGa heat-treated samples were analysed for the presence of oligomers, aggregates and possible cross-links under reducing and non-reducing SDS/PAGE conditions (Figure 5). Under non-reducing SDS/PAGE conditions bands corresponding to monomeric and dimeric β-LGa protein

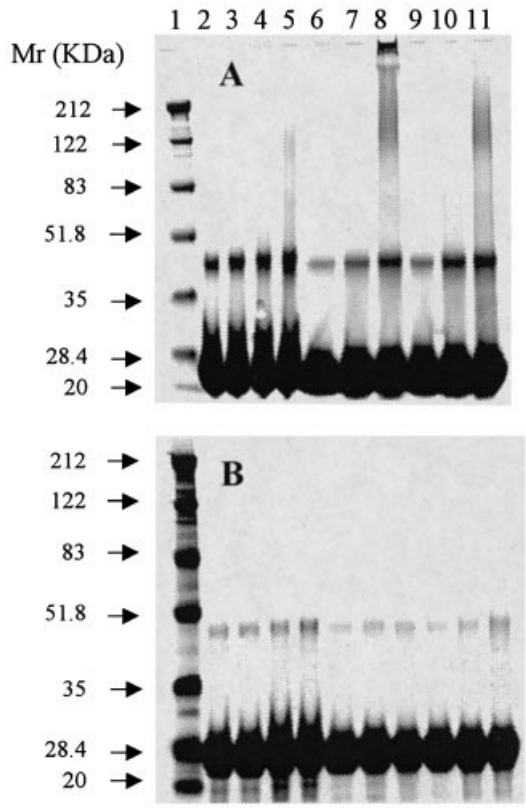


Figure 5 SDS/PAGE analysis of β-LGa samples heat treated at 60 °C under non-reducing (A) and reducing (B) conditions

Lane 1, molecular-mass markers; lanes 2–5, samples formulated in 1.75 M (60%) sucrose, 1.5 M glycine and 10 mM lysine and heat-treated for 0 h (lane 2; unheated control), 10 h (lane 3), 72 h (lane 4) and 144 h (lane 5); lanes 6–8, samples formulated in 50 mM glucose, 60% sorbitol, 1.5 M glycine and 10 mM lysine and heat-treated for 10 h (lane 6), 72 h (lane 7) and 144 h (lane 8); lanes 9–11, samples formulated in 50 mM fructose, 60% sorbitol, 1.5 M glycine and 10 mM lysine and heat-treated for 10 h (lane 9), 72 h (lane 10) and 144 h (lane 11).

forms were prevalent in all samples (Figure 5A), including unheated control samples (Figure 5A, lane 2). It was necessary to overload the sucrose heat-treated samples relative to glucose- and fructose-formulated and heat-treated samples in order to clearly visualize the dimeric band (Figure 5A, lanes 2–5). A correlation between the length of heat treatment and the relative intensity of the dimeric band was clearly evident in all formulations, with longer heat treatments resulting in a more intense dimeric band (Figure 5A). Increasing the anti-viral heat treatment to extreme levels (60 °C, 144 h) resulted in the formation of higher-molecular-mass aggregates that were observed as smeared bands (Figure 5A, lanes 5, 8 and 11). Aggregate material that could not penetrate the gel was also observed in glucose-formulated samples (Figure 5A, lane 8). Under reducing conditions the electrophoretic pattern of the β-LGa heat-treated samples changed dramatically and monomer bands

were predominant (Figure 5B). These results indicate that the majority of the dimeric and aggregate bands observed under non-reducing conditions were due to disulphide cross-links. However, bands corresponding to dimeric protein forms were observed in all reduced samples, albeit with an intensity of only a few per cent of the non-reduced samples, whereas bands corresponding to higher-molecular-mass oligomers and aggregates were not present in any of the heat-treated samples after reduction (Figure 5B). Although the intensity of the reduced dimeric bands was weak, the intensity clearly increased with extended lengths of heat treatment, indicating that a small proportion of the β-LGa dimers was not due to disulphide-bonded cross-links. Image analysis of the reduced and non-reduced SDS/PAGE gels shown in Figure 5 revealed that, for samples formulated in sucrose and heat-treated for 144 h, the non-disulphide cross-linked component of β-LGa dimers was approx. 7.5% of the total dimer population. In the case of glucose-formulated samples non-disulphide cross-linked dimers contributed to approx. 3% of the total dimer population after 144 h, while for fructose-formulated samples non-disulphide cross-linked dimers made up 9.2% of the total dimer population after heat treatment for 144 h.

Discussion

Covalent modification of β-LGa during anti-viral heat treatment in the presence of sucrose as a thermostabilizing excipient

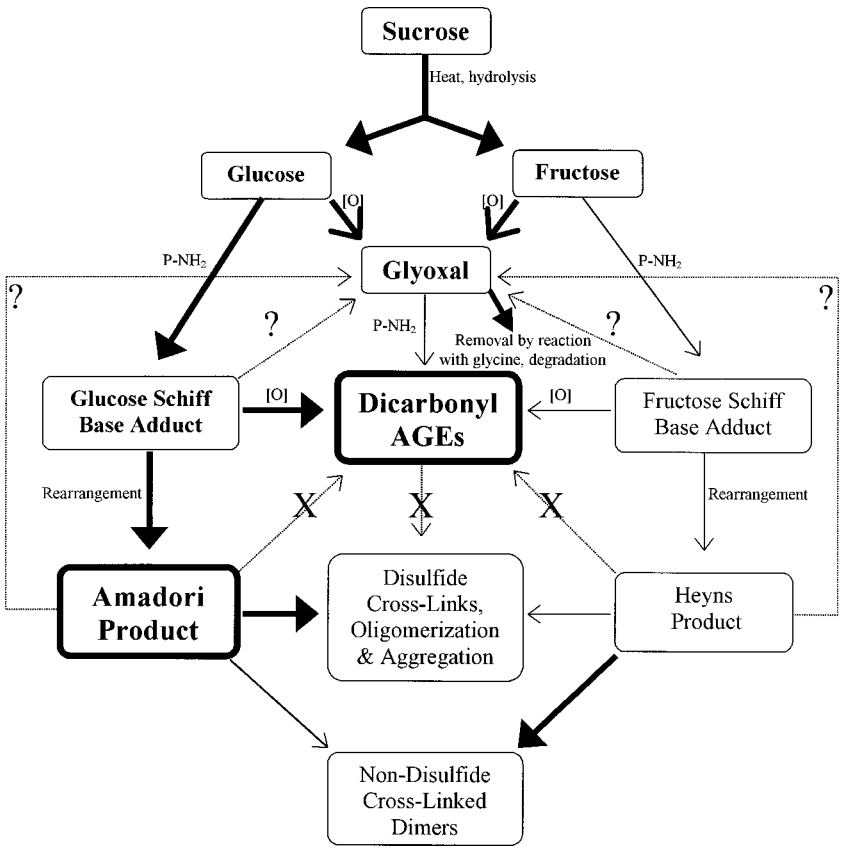
Detection and unequivocal assignment of glycation products during heat treatment was relatively straightforward due to the clear and concise mass shift (162 Da) associated with such modifications. However, assignment of the AGE products that accumulated with heat treatment was much more difficult, owing to the complex nature of these peaks and the numerous products that were theoretically possible. Of the currently described AGE adducts only two fitted the observed mass shift of approx. 56 Da corresponding to the major AGE product detected in β-LGa heat-treated samples, these being CML and methylglyoxal hydroimidazolone. In view of the fact that significant quantities of methylglyoxal are not present in the β-LGa heat-treated samples (Figure 3), and that the observed modifications occur at lysine residues, we conclude that the major AGE modification detected in β-LGa heat-treated samples is most likely to be CML.

As glycation products and their intermediates are known to undergo further reactions and degradations to yield AGEs, a major objective of the current study was to elucidate the mechanisms leading to such covalent modifications during heat treatment in standard sucrose bioprocessing formulations, with a view to minimizing such potentially damaging reactions. In consideration of the data presented in this study we propose the mechanism outlined in Scheme 1 for the routes leading to the formation of the β-LGa covalent adducts observed upon heat treatment in the presence of sucrose. Initially, sucrose is hydrolysed to yield relatively low concentrations of glucose and fructose, which then react with the more reactive amino groups on the protein surface to ultimately form Amadori and Heyns products respectively (Scheme 1). The greater reactivity of glucose means that most of the early glycation products are derived from reaction of the protein with glucose, that is to say they are predominantly Amadori products formed via the glucose Schiff-base adduct (Scheme 1). Although we found no evidence for the reaction of reducing sugars or their autoxidation products at other amino acids, we cannot rule out the possibility that small, undetectable populations of such adducts exist.

Associated with the glycation of β-LGa during extended heat treatment is an increase in the formation of AGEs giving a net increase of approx. 56–58 Da per AGE adduct. Our data show clearly that oxidative conditions are required for the formation of such adducts, which could, in theory, be formed from three possible oxidative routes. The first of these is the modification of β-LGa by direct reaction with 1,2- dicarbonyl compounds (glyoxal) arising from sugar autoxidation. Our results show clearly that glyoxal was formed at approximately equal rates from both glucose and fructose under the bioprocessing conditions utilized, and the reaction of glyoxal with β-LGa did indeed yield protein adducts consistent with the condensation of glyoxal with lysine amino groups. Yet, if glyoxal was the sole or major source of the AGE products observed upon heat treatment of sucrose, glucose and fructose samples we would expect the yield of these adducts to be approximately the same in heat-treated β-LGa samples formulated in glucose or fructose. This is clearly not the case (Figure 2), and we therefore conclude that this pathway is only a minor contributor to the formation of the observed AGE population.

The second possible route for the formation of the observed AGE adducts is from the direct breakdown of Amadori and Heyns products [8,20,25]. However, when the β-LGa Amadori and Heyns products formed upon heat treatment under anti-oxidative conditions (Figures 2C and 2D) were reformulated in sorbitol and pasteurized a second time under oxidative conditions, the AGE adducts previously observed upon heat treatment of glucose- and fructose-formulated samples under oxidative conditions were not reproduced (Figures 2E and 2F). This is compelling evidence that the majority of the population of AGE products observed upon anti-viral heat treatment is not derived from the breakdown of Amadori or Heyns products.

The third possible route for the formation of the β-LGa AGE adducts is the degradation of the Schiff-base adduct by an amine-assisted oxidative step prior to the formation of



Scheme 1 Mechanistic routes of protein glycation, AGE adduct formation and protein oligomerization during anti-viral heat treatment of β-LGa samples

Anti-viral heat treatment was at 60 °C formulated in 1.75 M sucrose, 1.5 M glycine and 10 mM lysine, pH 7. Major products and pathways leading to the formation of AGEs and glycation adducts are highlighted in **bold**. Normal arrows indicate pathways that contribute minimally to the formation of protein adducts. Dotted arrows with crosses represent pathways that were not activated in the current study. Dotted arrows with question marks represent pathways whereby we could not determine whether they were activated during heat treatment. P-NH_2, protein amino group; [O], oxidation.

the more stable glycation adduct [23]. Unfortunately it is not possible to isolate such Schiff-base adducts for study as they are either converted rapidly into the more stable glycation products or are hydrolysed during attempts to purify them [9]. However, in view of the fact that the other possible mechanistic routes do not play a major role in AGE formation under the conditions utilized in this study, we propose that it is from the Schiff-base adducts that the majority of AGE products observed in this study are derived (Scheme 1). Furthermore, Glomb and Monnier [23] have argued that the Schiff base is a primary intermediate in the formation of CML. Consequently we propose that the major mechanism of AGE formation under the heat-treatment conditions used in this study is via degradation of the glucose-derived Schiff-base adduct (Scheme 1).

β-LGa aggregation during heat treatment

The loss of solubility and aggregation of β-LGa upon heat treatment above 60 °C due to denaturation and disulphide-linked polymerization in the absence of thermostabilizing agents is well documented [15,26–29]. Likewise, the formation of covalently cross-linked protein aggregates as a result of glycation has also been studied and reported extensively [10,30–34], although the mechanisms and processes by which these reactions proceed is poorly understood. In the current study we have shown that increases in the dimeric and aggregate forms of β-LGa with heat treatment are primarily due to the formation of disulphide-linked oligomers and aggregates. β-LGa has a free thiol group (Cys^{121}) [15] and it is presumably via this group that the dimeric, oligomeric and aggregate disulphides are cross-linked. This is consistent with work carried out previously in this laboratory whereby heat-treated samples of lysozyme, which contains no free thiol groups, did not lead to the formation of dimeric or aggregate material upon protein glycation [7].

Although glycation does not appear to play a significant

role in the formation of the majority of the observed β-LGa dimers and aggregates during heat treatment, it has been proposed previously that glycation and oxidative damage play a role in sulphur-group oxidation leading to and enhancing the formation of disulphide-bonded protein aggregates [15,35]. Furthermore, the crystal structure of β-LGa shows that the free Cys thiol group is partially buried [36] and Morgan et al. [15] have postulated that the observed 'glycation' of β-LGa with lactose near the free thiol group results in the destabilization of some local secondary structure, increasing the exposure of the free thiol group and permitting an increase in thiol–disulphide interchange reactions. Our data suggest that such a mechanism could enhance the formation of the β-LGa dimers and aggregates observed in the present study. Tryptic peptide mapping in this laboratory has shown that, during heat treatment, β-LGa is preferentially glycated at lysine residues 100 and 101, these residues being situated in a di-lysine motif near the free Cys thiol in the three-dimensional protein structure (results not shown). We therefore propose that, in β-LGa samples subjected to extended heat treatments, preferential glycation at the di-lysine motif (residues 100–101) near the free Cys thiol may induce local secondary-structure destabilization permitting increased thiol–disulphide interchange, oligomerization and aggregation. The presence of glucose results in greater glycation at this di-lysine motif compared with fructose and therefore leads to more extensive aggregation (as observed by SDS/PAGE, Figure 5), relative to that observed with fructose-formulated samples (Scheme 1). Therefore in sucrose heat-treated samples the presence of relatively high concentrations of the glucose-derived Amadori products enhances the rate of formation of disulphide cross-linked oligomers and aggregates. Furthermore, samples heat-treated in the presence of sorbitol (which is not hydrolysed to reducing sugars) in place of sucrose resulted in lower levels of dimerization and aggregation consistent with glycation-induced enhancement of the oligomerization and aggregation pathways.

Despite disulphide-bonded dimers representing the majority of β-LGa cross-linked material after heat treatment, low-intensity dimer bands remained in all samples (heat-treated and unheated controls) even under reducing conditions (Figure 5B). The intensity of non-disulphide-linked dimers, although weak, increased with extended anti-viral heat treatments. This small population of non-disulphide cross-linked dimers observed under reducing conditions is most likely due to the presence of a small population of glycation-derived AGE cross-links that could not be detected by ESI-MS. The formation of protein AGE cross-links is known to be a relatively slow process [34,37,38], so even under the elevated temperatures used in this study (60 °C) only small concentrations of such adducts were observed. The increase in the proportion of non-disulphide linked dimers with extended heat treatment was elevated in fructose heat-treated samples relative to glucose heat-treated samples (Figure 5B). We tentatively suggest that this is a result of the greater reactivity of glucose relative to fructose under the conditions utilized in this study. Most currently characterized glycation-derived cross-links require an amino-group–amino-group cross-link. Therefore we propose that the greater reactivity of glucose results in the most accessible and reactive amino groups been 'tied up' by reaction with glucose, leaving fewer free amino groups available for glycation cross-linking reactions. In the case of the less reactive fructose, fewer glycation products are formed and thus there are many more β-LGa molecules with free, reactive amino groups available for reaction with fructose Heyns-product-modified β-LGa molecules to form cross-linked AGE dimers. Hence the relative concentration of glycation-derived cross-links appears to be elevated in fructose heat-treated samples compared with glucose heat-treated samples.

Concluding remarks

The data presented in this study confirm that anti-viral heat treatment of liquid β-LGa samples formulated in the presence of sucrose as a thermostabilizing excipient results in covalent modifications of the protein by glucose, and to a lesser extent by fructose and glyoxal. Although we have no evidence that these modifications are harmful themselves, recent reports link the presence of 1,2-dicarbonyls and AGEs to complications in heat-treated peritoneal-dialysis fluids [12]. Reddy et al. [39] have also reported that CML is a major AGE antigen recognized in tissue proteins by anti-AGE antibodies. Fortunately, the amount of protein modification during standard bioprocessing conditions (10 h) was relatively low, but covalently modified adducts were still present. Therefore, we recommend that actions be considered by those in the bioprocessing industry to introduce measures to limit or minimize such modifications further. This may involve avoiding the use of sucrose as a thermostabilizing reagent, minimizing the length of heat treatment and/or the introduction of further formulation components such as aminoguanidine, which is reported to trap 1,2-dicarbonyls. This study has also elucidated the mechanisms of β-LGa modification during anti-viral heat treatment in the presence of sucrose. It is now necessary to investigate whether such modifications take place during anti-viral heat treatment of therapeutic products, such as in the C2 domain of Factor VIII preparations to which epitopes for neutralizing antibodies have been mapped [40]. If such modifications are found to be prevalent this could have implications for the biopharmaceutical industry's approach to anti-viral heat-treatment bioprocessing.

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References

1 Pollmann, H. (1994) Infusionsther. Transfusionsmed. **21** (suppl. 1), 80–83
2 Peerlinck, K., Arnout, J., Di Giambattista, M., Gilles, J. G., Laub, R., Jacquemin, M., Saint-Remy, J. M. and Vermylen, J. (1997) Thromb. Haemostasis **77**, 80–86
3 Raut, S., Di Giambattista, M., Bevan, S. A., Hubbard, A. R., Barrowcliffe, T. W. and Laub, R. (1998) Thromb. Haemostasis **80**, 624–631
4 Laub, R., Di Giambattista, M., Fondu, P., Brackmann, H. H., Lenk, H., Saenko, E. L., Felch, M. and Scandella, D. (1999) Thromb. Haemostasis **81**, 39–44
5 Peerlinck, K., Rosendaal, F. R. and Vermylen, J. (1993) Blood **81**, 3332–3335
6 Knevelman, A., de Wit, H. J., Griffin, B., Hart, H. and McIntosh, R. V. (1994) Vox Sang. **66**, 96–103
7 Smales, C. M., Pepper, D. S. and James, D. C. (2000) Biotechnol. Bioeng. **67**, 177–188
8 Bucala, R. (1996) Diagn. Endocrin. Metab. **14**, 99–106
9 Suarez, G., Rajaram, R., Oronsky, A. L. and Gawinowicz, M. A. (1989) J. Biol. Chem. **264**, 3674–3679
10 Nagaraj, R. H., Shipanova, I. N. and Faust, F. M. (1996) J. Biol. Chem. **271**, 19338–19345
11 Vasan, S., Zhang, X., Kapurniotu, A., Bernhagen, J., Teichberg, S., Basgen, J., Wagle, D., Shih, D., Terlecky, I., Bucala, R. et al. (1996) Nature (London) **382**, 275–278
12 Schalkwijk, C. G., Posthuma, N., tenBrink, H. J., terWee, P. M. and Teerlink, T. (1999) Periton. Dialysis Int. **19**, 325–333
13 Wells-Knecht, K. J., Zyzak, D. V., Litchfield, J. E., Thorpe, S. R. and Baynes, J. W. (1995) Biochemistry **34**, 3702–3709
14 Morgan, F., Bouhallab, S., Molle, D., Henry, G., Maubois, J. L. and Leonil, J. (1998) Int. Dairy J. **8**, 95–98
15 Morgan, F., Leonil, J., Molle, D. and Bouhallab, S. (1999) J. Agric. Food Chem. **47**, 83–91
16 Morgan, F., Leonil, J., Molle, D. and Bouhallab, S. (1997) Biochem. Biophys. Res. Commun. **236**, 413–417
17 Bunk, D. M. (1997) Anal. Chem. **69**, 2457–2463
18 Lapolla, A., Fedele, D., Aronica, R., Garbeglio, M., D'Alpaos, M., Seraglia, R. and Traldi, P. (1997) Rapid Commun. Mass Spectrom. **11**, 1342–1346
19 Lapolla, A., Fedele, D., Plebani, M., Aronica, R., Garbeglio, M., Seraglia, R., Dalpaos, M. and Traldi, P. (1999) Clin. Chem. **45**, 288–290
20 Zigrovic, I., Kidric, J. and Horvat, S. (1998) Glycoconj. J. **15**, 563–570
21 Ahmed, M. U., Thorpe, S. R. and Baynes, J. W. (1986) J. Biol. Chem. **261**, 4889–4894
22 Frye, E. B., Degenhardt, T. P., Thorpe, S. R. and Baynes, J. W. (1998) J. Biol. Chem. **273**, 18714–18719
23 Glomb, M. A. and Monnier, V. M. (1995) J. Biol. Chem. **270**, 10017–10026
24 Khalifah, R. G., Baynes, J. W. and Hudson, B. G. (1999) Biochem. Biophys. Res. Commun. **257**, 251–258
25 Khalifah, R. G., Todd, P., Booth, A. A., Yang, S. X., Mott, J. D. and Hudson, B. G. (1996) Biochemistry **35**, 4645–4654
26 Prabakaran, S. and Damodaran, S. (1997) J. Agric. Food Chem. **45**, 4303–4308
27 Hoffmann, M. A. M. and vanMil, P. (1997) J. Agric. Food Chem. **45**, 2942–2948
28 Hoffmann, M. A. M., Sala, G., Olieman, C. and deKruif, K. G. (1997) J. Agric. Food Chem. **45**, 2949–2957
29 Qi, X. L., Holt, C., McNulty, D., Clarke, D. T., Brownlow, S. and Jones, G. R. (1997) Biochem. J. **324**, 341–346
30 Rahbar, S., Yernini, K. K., Scott, S., Gonzales, N. and Lalezari, I. (1999) Biochem. Biophys. Res. Commun. **262**, 651–656
31 Lederer, M. O. and Buhler, H. P. (1999) Bioorg. Med. Chem. **7**, 1081–1088
32 Thornalley, P. J. (1999) Clin. Lab. **45**, 263–273
33 Chellan, P. and Nagaraj, R. H. (1999) Arch. Biochem. Biophys. **368**, 98–104
34 Lee, K. W., Mossine, V. and Ortwerth, B. J. (1998) Exp. Eye Res. **67**, 95–104
35 Linetsky, M., James, H. L. and Ortwerth, B. J. (1999) Exp. Eye Res. **69**, 239–248
36 Qin, B. Y., Bewley, M. C., Creamer, L. K., Baker, E. N. and Jameson, G. B. (1999) Protein Sci. **8**, 75–83
37 Shamsi, F. A., Partal, A., Sady, C., Glomb, M. A. and Nagaraj, R. H. (1998) J. Biol. Chem. **273**, 6928–6936
38 Zhao, H. R., Nagaraj, R. H. and Abraham, E. C. (1997) J. Biol. Chem. **272**, 14465–14469
39 Reddy, S., Bichler, J., Wellsknecht, K. J., Thorpe, S. R. and Baynes, J. W. (1995) Biochemistry **34**, 10872–10878
40 Pratt, K. P., Shen, B. W., Takeshima, K., Davie, E. W., Fujikawa, K. and Stoddard, B. L. (1999) Nature (London) **402**, 439–442

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