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Evaluation of a sulphydryl–disulphide exchange index (SEI) for whey proteins—β-lactoglobulin and bovine serum albumin

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Abstract

Sulphydryl–disulphide (SH–SS) exchange underlies many protein functions in processed foods. There is a need for reliable indicators of SH–SS exchange capacity in protein ingredients. An index for SH–SS exchange (SEI) is described using β-lactoglobulin (BLG) or bovine serum albumin (BSA). The proteins were reacted with 2-pyridine disulphide (PDS Aldrithiol-2™) or Ellman's reagent (DTNB) in a buffered medium (0.05 M phosphate buffer, pH 6.9) at 25 °C. The 2nd order rate constant for protein SH–SS exchange with PDS or DTNB (k, M^{-1} s^{-1}) was normalized by dividing by the rate constant for glutathione (GSH) reaction with PDS or DTNB (k^*, M^{-1} s^{-1}) determined under identical conditions. The capacity or SH–SS exchange was inversely related to SEI ($= -\log k/k^*$) values of 4.11 for BLG and 1.05 for BSA, based on measurements using PDS. Studies using DTNB yield SEI equal to 4.28 for BLG and 2.20 for BSA. The SH group of BSA was 100–1052 times more reactive than the SH group of BLG. In a medium containing 1.2 M sodium chloride the difference in SH–SS exchange capacity was ~9000 fold. Differential scanning calorimetry (DSC) results showed that the conformational stability of BLG increased much more substantially than BSA when both proteins were exposed to 0.1–1.2 M sodium chloride concentrations.
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1. Introduction

The functional properties of whey proteins depend on their ability to form covalent and noncovalent interactions. The former consist of disulphide linkages formed by sulphydryl–disulphide (SH–SS) exchange (Hillier, Lyster, & Cheeseman, 1980). The requirements for SH–SS exchange are, a high total SH group concentration, usually measured in the presence of 8 M urea (Manning, Heinselman, Jennes, & Coulter, 1969), a high initial concentration of disulphide bonds per gramme of protein, and SH group unmasking from within proteins. Solvent properties also affect the rate of SH–SS exchange including, ionic strength, pH, and nature of ions, additives, and temperature (Calvo, Leaver, & Banks, 1993; Hillier et al., 1980; Howell & Taylor, 1995).

The total SH group concentration is unchanged during SH–SS exchange. Processes such as β-elimination or oxidation are directly responsible for SH group loss (Friedman, 1973). The functionality of protein ingredients is highly correlated with total SH concentration (Kim et al., 1988). In contrast, Hillier et al. (1980) found no direct correlation between free SH group concentration and the gelling time for reconstituted whey powders. With processed milk, SH–SS exchange leads to complexation of whey proteins and κ-casein which affects cheese and yogurt manufacture (Hill, 1989). There is a need for accurate measures of SH–SS exchange.

This paper describes the measurement of a SH–SS exchange index (SEI) using two simple disulphide compounds, pyridine disulphide (PDS Aldrithiol-2™) or Ellman's reagent, as surrogates for protein disulphide bonds (Apenten & Galani, 2000; Galani & Apenten, 2000). The SEI was determined for β-lactoglobulin (BLG) and bovine serum albumin (BSA). Each of these bovine whey proteins has one SH group. The reaction of glutathione (GSH) with PDS (or DNTB) is used to normalize results from different experiments. The effect of increasing ionic strength on the SEI was compared with NaCl effects on protein stability measured by differential scanning calorimetry (DSC).

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2. Materials and methods

2.1. General

BLG (Product no. L-3908; Lot no. 119H7008) was a mixture of genetic variants A and B; BSA (Product no. A-7906, Lot no. 22K1687), pyridine disulphide (PDS), urea (ultra-pure grade), NaCl, glutathione (γ-glutamyl-cystinyl-glycine; GSH) and all other chemicals were purchased from Sigma-Aldrich Ltd (US). Protein solutions (2.2×10^{-4} M) were prepared by dissolving BLG or BSA with sodium phosphate buffer (50 mM; pH 6.9). The solutions were centrifuged and actual protein concentrations were determined from absorbance readings at 280 nm. The extinction coefficients ($E^{1\%}{}_{1\ cm}$) for BLG and BSA were taken as 9.5 and 6.6, respectively. Solutions of PDS were prepared and standardized as described elsewhere (Apenten & Galani, 2000).

2.2. Spectrophotometry

Protein solution (0.1 ml; 2.2×10^{-4} M) was mixed with PDS (1.5 ml; 2.5×10^{-4} M) and NaCl (1.5 ml; 0–2 M). The rate of formation of 2-thio pyridine (2-TP) was monitored continuously at 343 nm as described before (Apenten & Galani, 2000). The total protein SH group concentration was determined from 343 nm readings taken in the presence of 8 M urea. Spectrophotometric readings were recorded with an α-Helios instrument coupled to a PC running Vision-Rate Software™ (Thermo Spectronic, New York, USA). For measurements using Ellman's reagent, we added DTNB solution (0.2 ml; 2.5 mM) to 2.7 ml of buffer containing 0.2 ml of protein solution (2.2×10^{-4} M). The rate of reaction was monitored at 412 nm for 1-h (BSA) or 16 h (BLG). All reactions were thermostated at 25 (±1)°C.

2.3. Calculation of a sulphydryl–disulphide exchange index

The SEI was determined from two 2nd order rate constants for PDS (or DNTB) reaction with protein (k) or GSH (k^*). The SEI is calculated from Eq. (1).

$$\mathrm{SEI}(=\log(k/k^*)) \quad (1)$$

2.4. Differential scanning calorimetry (DSC)

Weighed amounts of protein (10 mg) were dissolved with 100 μl buffer. Approximately 20 mg of (10% w/v) solution was sealed inside pre-weighed aluminium pans. The total weight of sample was determined by difference using a 5-place balance (Perkin-Elmer, Connecticut, USA). The DSC scans were run from 10 to 120 °C at a rate of 5 °C/min (Q100 DSC instrument; TA Instruments Ltd, USA).

3. Results and discussion

3.1. General

The SH–SS exchange contribution from individual whey proteins (WP) can be estimated *approximately* from the proportions of BLG and BSA in a sample. Bovine milk normally contains 3.2 g BLG and 0.4 g BSA per kg (Walstra, Geurts, Noomen, Jellema, & van Boekel, 1999). The BLG:BSA mole ratio is 29:1, taking the molecular weights for BLG and BSA as 18 400 and 66 000 Daltons, respectively. The *apparent* contribution of BLG and BSA to the total SH–SS exchange capacity for bovine WP is therefore 29:1. This value will change in accordance with the proportions of BSA and BLG present within different WP samples. For instance, WP prepared by ultrafiltration retains high levels of BSA whereas that produced by ion-exchange chromatography is virtually BSA-free. The concentration of BSA within milk also changes with mastitis and with the frequency or season of milking (Andrews & Anderson, 1979; Casper, Wendorff, & Thomas, 1998). A "concentration-based" estimate of SH–SS exchange capacity is also unsatisfactory, because this ignores differences in the *reactivity* of SH groups from different proteins.

3.2. Sulphydryl–disulphide exchange with PDS and Ellman's reagent

Two low molecular weight disulphide compounds were used as models for protein disulphide bonds. Table 1 shows a summary of the second order rate constants for reacting protein (k, $M^{-1}\ s^{-1}$) or GSH (k^*, $M^{-1}\ s^{-1}$) with PDS or Ellman's reagent. It can be seen that PDS was more reactive than Ellman's reagent. From data in Table 1, the ratio $k_{(PDS)}/k_{(DNTB)}$ was twenty-three for BSA, three for BLG and about two for GSH. PDS is a neutral molecule whereas Ellman's reagent is bi-anionic at pH ~7.0. In a low ionic strength solvent, charge–charge repulsions will decrease the rate of reaction between DTNB and any cysteine residue located within the proximity of a negatively charged amino acid residue, such as aspartate. On the other hand, a cysteine residue located within the proximity

Table 1
Second order rate constant for sulphydryl-disulphide exchange[a]

Sulphydryl compound	Rate constant ($M^{-1}\ s^{-1}$)		SEI*
	PDS	Ellman's reagent	
β-lactoglobulin	0.038 (±0.002)	0.014 (±0.00138)	4.11, 4.28
Bovine serum albumin	40 (±0.54)	1.74 (±0.063)	1.05, 2.20
Glutathione	570	270[b]	0, 0

[a] Reaction conditions: 0.05 M Phosphate buffer, pH 6.9 at 25 °C.
[b] Data from Zhang et al. (1998).
* SEI for PDS, PNTB.

of a positively charged group (lysine) would show increased reactivity with DTNB. Zhang, Le, and Means (1998) reported that k (M^{-1} s^{-1}) was equal to 4600 M^{-1} s^{-1} for the DTNB reaction with 2-mecaptoethylamine (net charge = +1) whilst k (M^{-1} s^{-1}) was 220 M^{-1} s^{-1} for reaction with 2-mercaptoethanol (Net charge = 0). The absence of charge–charge effects is an advantage during SH-group quantification using PDS (Reiner, Kada, & Gruber, 2002). In this study, using PDS and DTNB provides a more complete picture of the range of responses obtainable for SH–SS exchange.

3.3. Sulphydryl–disulphide exchange index (SEI) for BLG and BSA

Protein concentration and total SH group concentration are taken into account in calculations leading to k (M^{-1} s^{-1}) as described previously (Apenten & Galani, 2000). Values for k (M^{-1} s^{-1}) provide a preliminary estimate of the SH–SS exchange capacities of different proteins. Unfortunately, it is difficult to achieve high day-to-day precision when measuring k (M^{-1} s^{-1}) unless such experiments are done under highly controlled conditions of temperature, pH and ionic strength. By contrast, values of k/k^* should be less susceptible to small day-to-day or system–system changes in experimental conditions, provided that both rate constants are measured. The logarithmic function in Eq. (1) compresses the SEI scale. In summary, the SEI is a more reliable measure of the SH–SS exchange capacity than straight forward rate measurements.

During the tests using PDS as the model disulfide compound, the SEI was 4.11 for BLG and 1.05 for BSA. When Ellman's reagent was the model disulphide compound, we found that the SEI values for BLG or BSA were 4.28 or 2.19, respectively (cf. Table 1, last column). Compared to BLG, it is clear that BSA is 2–3 orders of magnitude more reactive towards SH–SS exchange.

Under non-denaturing conditions, SH–SS exchange involves surface-located protein SH groups. BSA has one surface SH group (Calvo et al., 1993; Hillier et al., 1980; Howell & Taylor, 1995). In 8 M there were 0.65 mols of (total) SH-group per mol BSA. This result agrees with previous reports and is ascribed to the formation of BSA-mixed disulphides with *oxidized* glutathione or cysteine from blood plasma (Wilson, Wu, Motiu-Degrood, & Hupe, 1980). By contrast, urea-denatured BLG had 1.00 mol total SH groups per mol protein. The free SH group of BSA is located at the solvent-exposed cysteine-34 in the N-terminal domain which, lacking disulphide bridges, is highly flexible (Batra, Sasa, Ueki, & Takeda, 1989). By comparison, the free SH group of BLG occurs at cysteine-121 which is buried behind an α-helix located at the subunit dimerization interface (Brownlow et al., 1997) and is therefore less reactive.

3.4. Effect of salt concentration on SH–SS exchange index

To illustrate the utility of the SEI, we examined the effect of increasing NaCl concentration on the reaction of PDS with BLG and BSA. A plot of SEI values at different salt concentrations is shown in Fig. 1. The data fitted straight-line equations: $SEI_{BSA} = 0.125$ [NaCl] + 1.04 and $SEI_{BLG} = 0.856$ [NaCl] + 4.11. The gradients of these lines reveal that the SEI value for BLG is seven-times more sensitive to NaCl concentration changes than that of BSA. In the presence of 1.2 M NaCl, the SEI value of BSA equals 1.19 compared with 5.14 for BLG. The difference in SH–SS exchange capacity for these proteins is therefore 9000-fold in a high-salt medium. Matsudomi, Rector, and Kinsella (1991) found that 0–40 mM NaCl increased BSA gelation by reducing intermolecular repulsion. In contrast, high NaCl concentrations of 80–400 mM inhibited BLG gelation. Protein gelation requires disulphide bond formation via SH–SS exchange as well as hydrophobic and electrostatic interactions (Lee & Hirosee, 1991). Based on present results, high NaCl concentrations will reduce the SEI for BLG. Moreover, such effects originate from the impact of salt on protein structure and/or protein stability (see later). Salt effects independent of protein structure should "cancel out" when values of k (M^{-1} s^{-1}) are normalized by division with k^*(M^{-1} s^{-1}), as shown in Eq. (1). Finally, the SEI will also not be too prone to local charge–charge effects because PDS is a neutral molecule.

3.5. Differential scanning calorimetry and the SEI values of proteins

As discussed earlier, addition of 1.2 M sodium chloride reduced the SH–SS exchange capacities of BLG and BSA by 10- and 1.14-fold. It seems that the nature of SEI calculations makes it likely that salt effects arise from changes in protein structure. To test this idea, we performed DSC studies for BSA and BLG in the presence of increasing concentrations of NaCl. The results shown in Fig. 2 and Table 2 reveal that increasing NaCl

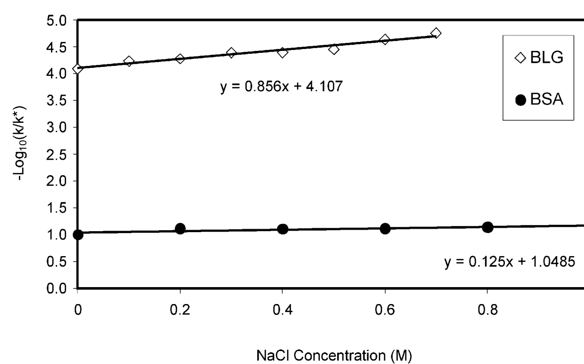

Fig. 1. Effect of NaCl concentration on the SH–SS exchange indices (SEI) of BLG and BSA.

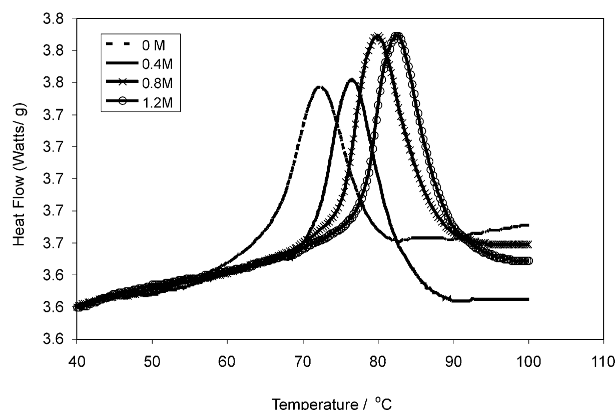

Fig. 2. Differential scanning calorimeter traces for β-lactoglobulin as a function of increasing salt (NaCl) concentration.

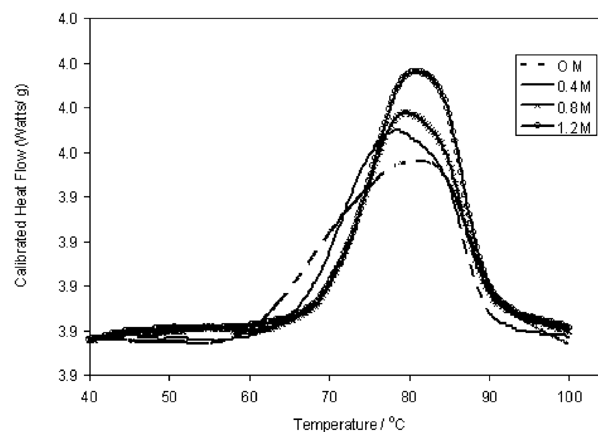

Fig. 3. Differential scanning calorimeter traces for bovine serum albumin as a function of increasing salt (NaCl) concentration.

Table 2
Thermal denaturation parameters for β-lactoglobulin and bovine serum albumin determined by DSC

Protein	Parameters	NaCl concentration (M)			
		0	0.4	0.8	1.2
BLG	Onset temperature (°C)	65.4±0.18	70.63	74.48	77.2±1.51
	Max. temperature (°C)	72.2±0.05	76.56	79.86	82.6±0.40
	$W_{1/2}$ (°C)	7.9±0.06	7.22	7.47	7.2±0.63
	Enthalpy (J/g)	13.2±0.58	14.42	15.38	17.3±1.26
BSA	Onset temperature (°C)	61.9 ±1.23	66.0	67.0	70.0 ±0.05
	Max. temperature (°C)	82 ±0.79	78.2	79.4	81.1 ±0.24
	$W_{1/2}$ (°C)	19.2 ±0.78	15.7	13.55	12.9±0.21
	Enthalpy (J/g)	19.4 ±1.27	18.0	17.3	20.5 ±1.48

concentrations stabilize BLG but have little or no effect on BSA. A more detailed analysis shows that the onset temperature for thermal denaturation, denaturation peak temperature and enthalpy for BLG denaturation all increase with increasing NaCl concentrations. This agrees with previous studies using urea as denaturant (Kella & Kinsella, 1988; Owusu-Apenten, 2002). By comparison, with increasing NaCl concentration, DSC peaks for BSA become progressively narrower and the peak onset temperature increases (Table 2; Fig. 3). However, the enthalpy of denaturation did not increase significantly over 0–1.2 M NaCl. It may be concluded that BSA denatures in a more cooperative fashion in a high salt medium though the net stability is not greatly affected.

High NaCl concentration does not affect the solvent exposed cysteine-34 of BSA. Modification of cysteine-34 with DNTB, iodoacetate or iodoacetamide did not affect the 2° structure of native BSA (66% α-helix, 33% random structure and 3% B-sheet). Nevertheless, the 3° structure of SH-modified BSA was apparently destabilized (Batra et al., 1989). By contrast, modification of cysteine-121 prevents the dimerization of BLG and produces large reductions in protein stability. Indeed, it is likely that cysteine-121-modified BLG is lacking 3°-structure as well as 4°-structure at room temperature (Ralston, 1972; Sakai, Sakurai, Sakai, Hoshino, & Goto, 2000). Also, well known is the fact that increasing salt concentration stabilizes the 3° and 4° structure of BLG. Such processes will lead to greater masking of cysteine-121. The SH group of BLG becomes exposed and activated towards PDS when the dimer dissociates to form two monomers (Apenten & Galani, 2000). Further destabilization with >4 M urea increases the SH-group reactivity still further, due to unfolding of BLG monomer (Apenten, 1998). Results reported in this paper reveal the opposite effect; namely, stabilization of BLG by NaCl reduces its capacity to undergo SH–SS exchange.

The SH–SS exchange index proposed is a useful tool for examining the likely role played by BLG and BSA in milk or WP ingredients. Based on current measurements of the SEI and the mole proportions of the two proteins found in normal milk, we expect that the contribution of BSA to SH–SS exchange will be ~30-times greater than BLG under the conditions used in this study. Shuffling SH and disulphide bonds begins with catalytic amounts of a reactive SH-group. The impact of BSA is also likely to increase in high ionic strength solvents. Further studies are needed to examine the effect of temperature and other factors on SEI of BLG and BSA. However, adopting a ratio k/k^* as the measure of SH/SS exchange cancels out the effect of extraneous effects on SH-group reactivity (as described earlier). This approach increases the reliability of the present SH–SS exchange measurements as compared with single rate measurements. More research is also needed to extend the present method to more "realistic" foods. At the current stage of development, the SEI can be determined for mixtures of food proteins, such as WP concentrate or isolates.

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