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# The roles of disulphide and non-covalent bonding in the functional properties of heat-induced whey protein gels

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Heat-induced gelation (80 °C, 30 min or 85 °C, 60 min) of whey protein concentrate (WPC) solutions was studied using transmission electron microscopy (TEM), dynamic rheology and polyacrylamide gel electrophoresis (PAGE). The WPC solutions (150 g/kg, pH 6·9) were prepared by dispersing WPC powder in water (control), 10 g/kg sodium dodecyl sulphate (SDS) solution or 10 mM-dithiothreitol (DTT) solution. The WPC gels containing SDS were more translucent than the control gels, which were slightly more translucent than the gels containing DTT. TEM analyses showed that the SDS-gels had finer aggregate structure (≅10 nm) than the control gels (≅100 nm), whereas the DTT-gels had a more particulate structure (≅200 to 300 nm). Dynamic rheology measurements showed that the control WPC gels had storage modulus (G′) values (≅13 500 Pa) that were ≅25 times higher than those of the SDS-gels (≅550 Pa) and less than half those of the DTT-gels after cooling. Compression tests showed that the DTT-gels were more rigid and more brittle than the control gels, whereas the SDS-gels were softer and more rubbery than either the control gels or the DTT-gels. PAGE analyses of WPC gel samples revealed that the control WPC solutions heated at 85 °C for 10 min contained both disulphide bonds and non-covalent linkages. In both the SDS-solutions and the DTT-solutions, the denatured whey protein molecules were in the form of monomers or small aggregates. It is likely that, on more extended heating, more disulphide linkages were formed in the SDS-gels whereas more hydrophobic aggregates were formed in the DTT-gels. These results demonstrate that the properties of heat-induced WPC gels are strongly influenced by non-covalent bonding. Intermolecular disulphide bonds appeared to give the rubbery nature of heat-induced WPC gels whereas non-covalent bonds their rigidity and brittle texture.

**Keywords:** Disulphide bonds, dithiothreitol, gelation, non-covalent bonding, sodium dodecyl sulphate.

Whey protein concentrates (WPCs) are used in a wide range of food applications not only because of their nutritional value but also because of their functional properties, such as the ability to form heat-induced gels. They are used as functional ingredients in many foods, such as processed meat, bakery and dairy products (Kinsella & Whitehead, 1989). However, commercial uses of WPCs are limited because of unpredictable variations in their functional properties, due to composition and processing inconsistencies (Xiong, 1992).

Two basic protein gel structures are commonly found in food systems (Doi, 1993). The first is a 'particulate' gel that is composed of relatively large particles loosely bound to one another to form a network. These gels are usually opaque and have poor water-holding capacity. The second is a 'fine-stranded' gel produced by association of strands or small diameter particles to form a network. These gels are usually translucent and have good water-holding capacity (Stading & Hermansson, 1991; Langton & Hermansson, 1992, 1996). Variations between these two types of gels exist and mixed gels are sometimes formed (Barbut, 1995). These two types of gel structures have also been observed in WPC gels (Aguilera, 1995). The formation of one or the other type is determined largely by differences between the rate of protein denaturation and the rate of aggregation of denatured proteins (Taylor & Fryer, 1992), and the relative proportions of protein–protein attractive and repulsive interactions (Egelandsdal, 1980; Clark & Lee-Tuffnell, 1986; Mulvihill & Kinsella, 1987; Gault & Fauquant, 1992). The formation of a fine-stranded gel requires that the rate of protein denaturation is faster than the rate of protein aggregation, and that there is a balance between the protein–protein attractive and repulsive interactions. Particulate

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gels are formed when the rate of aggregation of proteins is faster than the rate of protein denaturation, and the protein–protein attractive and repulsive interactions are not in balance. Excessive attraction results in a randomly aggregated coagulum or even precipitate, whereas excessive repulsion results in weak or non-existent aggregation (Tang et al. 1995).

The relationships among these variables and the functional properties of heat-induced whey protein gels are not fully understood. In order to improve the use of WPC products as functional ingredients, a better understanding of how these factors relate to the functional and textural properties of heat-induced WPC gels is required.

When WPC solutions (e.g. >80 g protein/kg, pH 6·9) are heated at a sufficiently high temperature (e.g. 75 °C), the protein molecules unfold and interact to form intermediate aggregates prior to the formation of a gel network (Aguilera, 1995; Havea et al. 1998). The formation of intermediate aggregates involves two broad types of bonding: covalent and non-covalent bonding. The former consists of inter- and intramolecular disulphide bonds (Gupta & Reuter, 1992) formed via sulphydryl–disulphide interchange or sulphydryl oxidation reactions (Monahan et al. 1995). The latter are non-covalent interactions, such as hydrophobic, hydrogen bonding, ionic and other weak interactions that also contribute to the formation of aggregates and a gel network (Mangino, 1992; McSwiney et al. 1994a, b). Although these studies showed that the heat-induced gelation of whey proteins involves these two types of bonds, the relative contribution of each type of bonds to the overall functional properties of heat-induced WPC gels remain unclear.

Alting et al. (2000) studied the roles of these types of bonds in acid induced gels of preheated whey protein isolates (WPI) solutions. They suggested that the initial microstructure of the gels was primarily determined by the acid-induced non-covalent interactions. Additional covalent disulphide bonds formed during gelation were involved in stabilising the network and increase gel strength. Studies of model protein systems, such as β-lactoglobulin in aqueous solutions, (Hoffmann & van Mil, 1997; Kitabatake et al. 2001) have thrown some light on the situation. These systems are quite different because of the lack of the strong gelling effect of α-lactalbumin and the large globular whey proteins (Gezimati et al. 1996; 1997). Likewise, conclusions drawn from studies on single protein cannot be applied to whey or WPC systems without modification.

The thiol-blocker, N-ethylmaleimide (NEM), has been commonly used in studies of protein interactions to prevent or slow down the thiol-catalysed disulphide interchange reaction (e.g. Hoffmann & van Mil, 1997; Alting et al. 2000; Kitabatake et al. 2001). The work reported here was an attempt to use another, rather obvious approach of adding small quantities of dithiothreitol (DTT) to the heated WPC solutions. DTT is a powerful reducing agent, and in WPC solutions it would cleave the disulphide bonds within the protein molecules as well as any intermolecular disulphide bonds. The globular proteins are therefore partially denatured and are held together by non-covalent associations, and during heat treatment, they are expected to be fully unfolded. The gelation process is expected involve non-covalent bonding only.

SDS is a powerful detergent and its overall effect on the protein is that it binds strongly to regions that are hydrophobic and carry a positive charge. In the heated protein systems, the protein molecules would be negatively charged hence avoiding all the non-covalent associations, so the protein-protein interactions are expected to be disulphide-linkages only.

## Materials and Methods

### *Materials*

A commercial spray-dried WPC powder, derived from mineral acid whey, was obtained from NZMP, Fonterra Co-operative Group (formerly Anchor Products), Edgecumbe, New Zealand. This WPC was typical of a standard commercial product. Analysis showed that the WPC powder had 850 g total protein/kg, 55 g fat/kg and 42 g moisture/kg. Mineral analyses showed that the powder contained 1·7, 13·8, 8·0 and 2·5 g/kg of calcium, potassium, sodium and phosphorus respectively.

The chemicals used for the preparation of the electrophoresis buffers (obtained from Bio-Rad Laboratories, Richmond, CA 94804, USA) were of analytical grade.

### *WPC composition*

The total protein content of the WPC powder was determined using the Kjeldahl method (AOAC, 1984), with a nitrogen conversion factor of 6·38. The fat content was determined using the Soxhlet extraction method, as described by Russell et al. (1980). The moisture content was determined by oven drying pre-weighed duplicate samples at 105 °C for 24 h, cooling in a desiccator for 2 h and re-weighing the samples. The mineral analyses were carried out at the New Zealand Pastoral Agricultural Research Laboratory, Palmerston North, by inductively coupled argon-plasma emission spectrometry using the method described by Lee et al. (1986).

### *Preparation of WPC solutions*

Appropriate quantities of WPC powder were dissolved in water (purified using a Milli-Q system, Millipore Corp., Bedford, MA 01730, USA; control), 10 mM-DL-dithiothreitol (DTT) or 10 g SDS/kg solutions, so that the final solutions contained 150 g WPC/kg. The protein concentration in these solutions was approximately 128 g/kg. The solutions were stirred for 2 h at room temperature using a magnetic stirrer and the pH was adjusted to 6·9 using 0·1 M-NaOH or HCl.

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*Preparation of heat-induced WPC gels*

The WPC solutions were placed in 400-cm-long, 30-mm-diameter, medium-walled polycellulosic plastic tubes and the ends were closed off using rubber bands to make stiff 'sausages'. These sausages were then placed in a thermostatically controlled water bath (85 °C±0·5) for 60 min. It took approximately 44 s to heat the middle of each tube to 84·8 °C. After heating, the tubes were removed from the water bath and immediately placed in an ice bath (≅0 °C) for 30 min before storing at 4 °C overnight. The gels were then analysed using a range of techniques.

The gels made from the control WPC solutions are referred to as 'control gels' in the text. The gels made from WPC solutions containing SDS or DTT are referred to as 'SDS-gels' or 'DTT-gels' respectively.

*Assessment of the gel clarity*

The WPC gels were removed from the tubes, and 3-mm-thick slices were cut from each. A slice of each gel was then placed on top of a bold black letter 'A', which had been printed on a transparent sheet, and then placed on top of an illuminant light box. The visibility of the printed letter as viewed through the slice was used as an indication of the clarity of the gels when compared with each other. The gels were then photographed in this set-up.

*Transmission electron microscopy*

Samples of the WPC gels were fixed, stained and photographed using a transmission electron microscope (TEM) as described by Langton & Hermansson (1996).

*Compression test*

Each of the WPC gel types was cut to give six cylindrical slices, each 25 mm in length, using a wire cutter and a template. Each slice was wrapped with plastic film to prevent moisture loss, placed in a sealed container and then left in a thermostatically controlled (20 °C) test room for 2 h to equilibrate before testing. The samples were placed between the upper 60 mm-diameter Teflon plate and the lower 95 mm × 105 mm Teflon plate of a TA-HD Texture Analyser (Stable Micro Systems, Haslemere, UK). The surfaces of the plates were lubricated with paraffin oil to minimize friction. The samples were then compressed to 80% of their original height at a rate of 0·83 mm $s^{-1}$ using a load cell of 500 N. The fracture stress and fracture strain were calculated for each sample using a software package based on analysis methods described by Truong & Daubert (2001).

The results were plotted in a texture map as described by Hamman & MacDonald (1992), and Truong & Daubert (2001), in order to provide information on the gel textures.

*Dynamic rheological measurement*

A set of WPC solutions was prepared and then heated in the 25 mm cup and bob configuration of a PARR PHYSICA US200 rheometer. The gap between the cup and the bob was 1 mm. A 13 ml sample of each WPC solution was placed in the cup. The top of the solution was about 1 mm above the top of the bob. A layer of paraffin oil was placed on top of the solution to avoid evaporation during heating. The changes in the viscoelastic properties of the WPC solutions were monitored during heating using the rheometer in oscillatory mode at a frequency of 1 Hz and a shear strain of (0·01). The solutions were heated from 20 to 80 °C at a rate of 1 deg C $min^{-1}$, held at 80 °C for 30 min, cooled from 80 to 20 °C at 1 deg C $min^{-1}$ and finally held at 20 °C for 20 min. The storage modulus, G′, and the loss modulus, G″, of the heated WPC solutions were measured every minute during the heating cycle. At the end of the 20 min holding at 20 °C, a frequency sweep (from 10 to 0·1 Hz) followed by a strain sweep (from 0·001 to 0·10) were conducted on the WPC gels, to ensure that the conditions of measurement were within the range of viscoelastic behaviour. Each WPC solution was tested in duplicate, at least.

*Polyacrylamide gel electrophoresis*

A set of WPC solutions was prepared and heated (85 °C), as described above, for 10 min instead of the 60 min used for gel formation. Samples (0·25 ml) of the solutions were then removed from the tubings, diluted with appropriate buffer and analysed using either alkaline-PAGE or SDS-PAGE as described by Havea et al. (1998). After preparing the appropriate gel, 10 µl samples of 0·10 g protein/kg solution were injected into the sample wells and then electrophoresed to separate the proteins. After the gels had been stained with Amido black dye and destained, they were photographed, as described by Havea et al. (1998).

## Results

*Clarity of the heat-induced WPC gels*

The control heat-induced WPC gel was partially translucent (Fig. 1a). The printed letter 'A' underneath the 3-mm-thick gel slice could be seen, although not clearly. Comparatively, the SDS-gel (Fig. 1b) was much more translucent. The dark letter underneath the gel slice could be seen clearly from the top. In contrast, the DTT-gel (Fig. 1c) was comparatively less translucent. The letter underneath the gel slice appeared like a 'shadow' without defined outlines.

*Analysis of heat-induced WPC gels using TEM*

The TEM photomicrograph of the control gel (Fig. 1a) showed a fine-stranded structure (≅100 nm) with some regions that were particulate, similar to the microstructure

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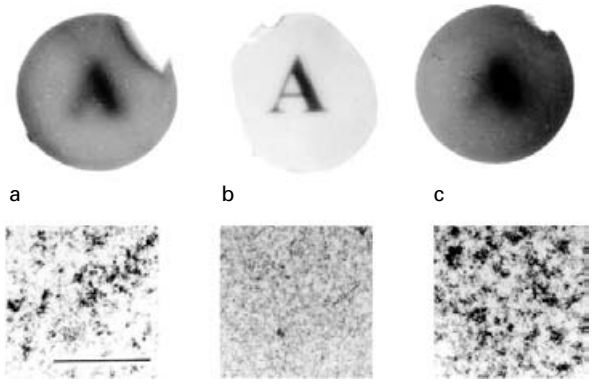


**Fig. 1.** Turbidity and TEM micrographs of heat-induced WPC gels; 150 g WPC/kg solutions, pH 6·9, heated at 85 °C for 60 min: a, control gel; b, SDS-gel; c, DTT-gel. To compare the opacities of the gels, photographs were taken of 3-mm-thick gel slices placed on top of printed black letters 'A'. The black bar in TEM micrograph a, is equivalent to 1 μm. All the TEM photomicrographs were of the same magnification (32 000 ×).

of heat-induced β-lactoglobulin gels reported by Stading et al. (1993). The SDS-gel (Fig. 1b) had much more evenly distributed fine-stranded structures (≅10 nm). In contrast, the DTT-gel (Fig. 1c) had a higher proportion of particulate structures (≅200–300 nm) than the control gel. These results were consistent with the clarity results (Fig. 1) because the fine-stranded structures of the SDS-gels allowed good light penetration, resulting in translucent gels. In contrast, the more particulate structures of the control gels and the DTT-gels restricted the penetration of light; hence they were more opaque.

### *Development of WPC gels during heating*

The formation of the WPC gels during heating was observed by recording the continuous changes in the viscoelastic properties of the WPC solutions at small deformation, thus avoiding the problems associated with gel fracture during measurement. The storage modulus, G′, which is a measure of the gel rigidity, and the loss modulus, G″, which is a measure of the gel viscosity, were recorded every minute during the heating cycle (Fig. 2). This technique has been used by many workers in studying the development of protein gels during heating (e.g. Tang et al. 1993; McSwiney et al. 1994a, b; Gezimati et al. 1996; Gezimati et al. 1997; Verdhanabhuti et al. 2001). The initial liquid systems typically have a G′ of zero and a low value of G″ (McSwiney et al. 1994a, b). At the gel point, G′ begins to rise, indicating the formation of a gel network. Therefore, gel point is defined as the time or temperature at which a measurable value of G′ is achieved.

The changes in G′ and G″ are shown in Fig. 2, with specific points of interest summarized in Table 1. When the control WPC solutions were heated from 20 to 80 °C, there were no measurable changes in the G″ values

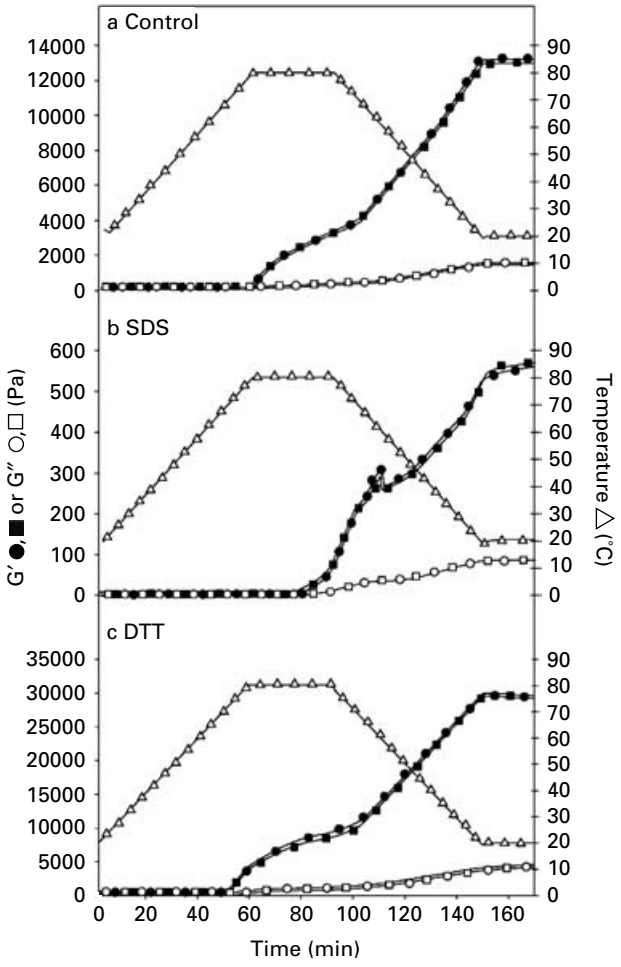


**Fig. 2.** Changes in the rheological properties of the WPC solutions during heating. 150 g WPC/kg solutions, pH 6·9, were heated (Δ) from 20 to 80 °C at 1 deg C $min^{-1}$, held at 80 °C for 30 min, cooled from 80 to 20 °C at 1 deg C $min^{-1}$ and held at 20 °C for 20 min. G′ (●, ■) and G″ (○, □) were measured every minute (the plotted data, from duplicate runs, are representative only, i.e. every fifth point). Measuring conditions: oscillatory mode, frequency of 1 Hz, strain of 0·01, cup and bob configuration.

(Fig. 2a), whereas G′ started to increase (i.e. gel point, Table 1) when the temperature reached 78 °C. During the 30-min holding at 80 °C, G′ continued to increase, reaching ≅3200 Pa at the end of the 30-min period. Meanwhile, no measurable changes in G″ value were achieved. Upon cooling from 80 to 20 °C, G′ continued to increase, reaching a value of ≅13 300 Pa, whereas the G″ value slowly increased, reaching ≅1450 Pa when the temperature reached 20 °C. During the second holding phase (20 °C), there were no measurable changes in either G′ and G″ values (Fig. 2a).

When the WPC solution containing 10 g SDS/kg was heated from 20 to 80 °C, there were no changes in the values of G′ or G″ (Fig. 2b). Unlike the control solution,

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**Table 1.** Viscoelastic properties of WPC solutions during heating, see profiles in Fig. 2

Values are means ± SD, for $n = 5$

| Properties | Control | SDS | DTT |
|---|---|---|---|
| Gel point (°C) | 78 ± 0·5 | 80 ± 0·5 | 63 ± 0·5 |
| Gel point (min) | 58 ± 0·5 | 80 ± 0·5 | 43 ± 0·5 |
| $G'_{80}$ (Pa) | 3220 ± 10 | 76 ± 2 | 8585 ± 205 |
| $G''_{80}$ (Pa) | 237 ± 4 | 13 ± 1 | 797 ± 16 |
| $G'_f$ (Pa) | 13 300 ± 200 | 555 ± 4 | 29 050 ± 450 |
| $G''_f$ (Pa) | 1450 ± 10 | 88 ± 2 | 4170 ± 340 |

$G'_{80}$ and $G''_{80}$ are values of G′ and G″ at the end of 30 min of holding at 80 °C
$G'_f$ and $G''_f$ are values of G′ and G″ after the heating cycle (i.e. after the final holding at 20 °C)

when the solution was held at 80 °C, the values of both G′ and G″ showed no measurable changes until after ≅20-min holding (i.e. gel point, Table 1), when G′ started to increase, reaching ≅50 Pa, whereas G″ slowly increased, reaching ≅13 Pa at the end of the 30-min holding period. During the cooling phase, G′ increased to ≅300 Pa, and then dropped by ≅50–100 Pa before it continued to rise again, reaching ≅450 Pa by the end of the cooling phase. (It is not clear why this drop in G′ occurs during the cooling phase, but this trend has been consistently observed in several replicate samples). Meanwhile, G″ slowly increased, reaching ≅75 Pa by the end of the cooling phase. During the final holding phase, G′ continued to rise slowly, reaching ≅550 Pa by the end of the holding time, whereas G″ remained constant.

When the WPC solutions containing DTT (Fig. 2c) were heated, G′ started to increase when the temperature reached 63 °C (i.e. gel point, Table 1), reaching ≅3500 Pa at the end of the heating phase. During the 80 °C holding, G′ continued to increase, reaching ≅9000 Pa (Table 1) at the end of the holding phase. Meanwhile, G″ started to increase slowly. During the cooling phase, the G′ value increased to ≅29 000 Pa and then remained constant during the second holding phase. The G″ value started to increase slowly only during the cooling phase, reaching about 4100 Pa, and then remained constant during the second holding phase.

Overall, the three WPC systems exhibited very different gelation behaviours. The control gels had G′ values that were ≅25 times higher (≅13 500 Pa) than those of the SDS-gels (≅550 Pa) after the heating cycle. In contrast, the DTT-gels had G′ values (≅29 000 Pa) that were more than double those of the control gels and ≅50 times higher than those of the SDS-gels. The gel point of the control WPC solutions occurred just before 80 °C was reached, whereas that of the solutions containing DTT occurred much earlier (63 °C) and that of the solution containing SDS occurred at a much later stage (after 20 min at 80 °C). The increases in G′ values occurred at fast rates during the cooling phase. During holding at 20 °C, there were minimal changes in the values of G′ in all of the WPC gels.

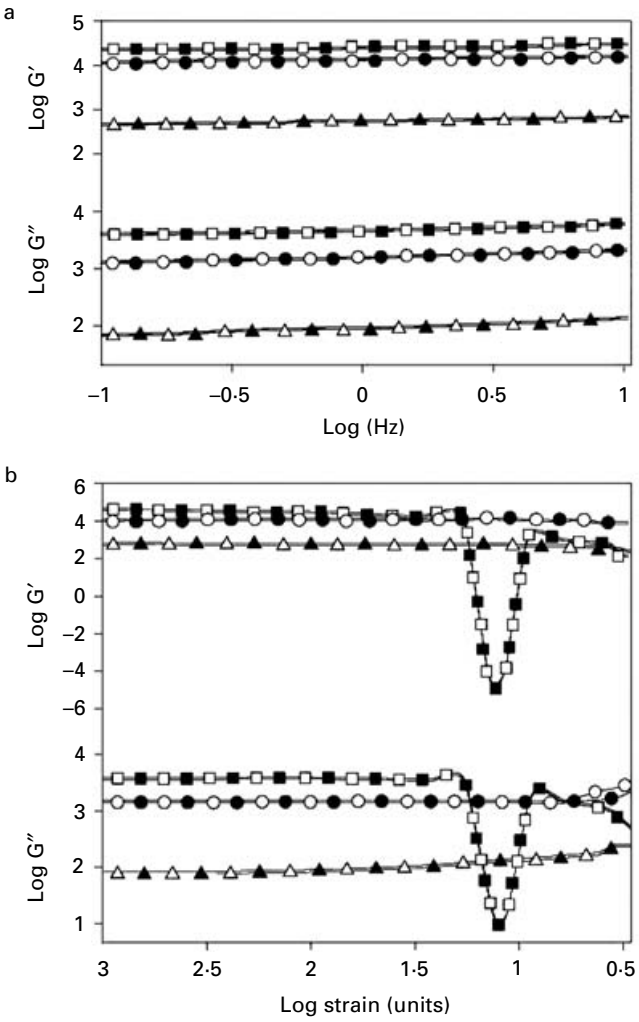


**Fig. 3.** Plots of (a) the frequency sweep (from 10 to 0·1 Hz) and (b) the strain sweep (from 0·001 to 0·10) of the heat-induced WPC gels after the heating cycles (see Fig. 2 for details of the heating cycles). Log G′ and log G″ were plotted against (a) log (frequency) and (b) log (strain), (○, ●) control gels (duplicate runs), (□, ■) DTT-gels (duplicate runs), (△, ▲) SDS-gels (duplicate runs).

### *Frequency and amplitude sweeps*

Frequency sweep (from 10 to 0·1 Hz) was carried out on the WPC gels after the heating cycle while measuring the values of both G′ and G″. The plots of log (frequency) versus log G′ or log G″ gave straight lines for all of the WPC gels (Fig. 3a). This indicated that the heat-induced WPC gels had elastic behaviour (no or limited relaxation) at the frequency range tested. It is clear that the selected frequency (1 Hz) used in the rheological measurements was within the linear range of the WPC gels. It should be pointed out that the responses of both the control gels and the DTT-gels to the changing sweep were at similar levels (≅4·0–4·5) and were higher than the levels (≅2·5–2·7) at which the SDS-gels responded to the changing frequencies.

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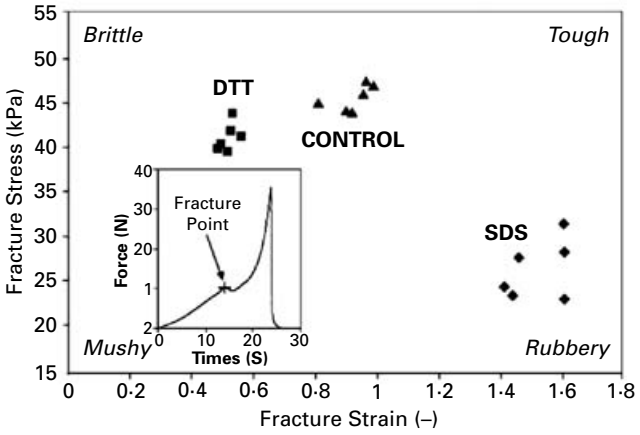


**Fig. 4.** Texture profile mapping of the heat-induced WPC gels; 150 g WPC/kg solutions, pH 6·9, heated at 85 °C for 60 min: control gel (▲), SDS-gel (◆) and DTT-gel (■). The inset shows a typical stress–strain plot.

This suggested that the DTT-gels probably had properties that were closer to those of the control gels than to those of the SDS-gels.

An amplitude (strain) sweep (from 1 to 100%) was also conducted on each of the gels. The plots of log (strain) versus log G′ or log G″ (Fig. 3b) for each gel also gave straight lines. It is clear that the G′ and G″ values for the DTT-gels dropped at log strain of ≅–1·3 (0·10), indicating that these gels were broken at this level of strain. This suggested that the DTT-gels were brittle compared with the control gels and the SDS-gels. Application of shear strain at levels higher than this would break the gels. The selected strain (0·010) used for the rheological measurement of these gels was within their linear range. Again, the responses of the control and DTT-gels to the changing strain (other than the breaking of the DTT-gels) were at similar levels and were at higher levels than those at which the SDS-gels responded.

### *Compression tests*

Heat-induced gels (150 g WPC/kg, pH 6·9, 85 °C for 60 min) were also analysed using compression tests. The fracture stress and strain data obtained from the compression test of each gel (Fig. 4, inset) were plotted in a texture map (Fig. 4, Truong & Daubert, 2001). The control gels fractured at similar stress levels but higher strain levels than that at which the DTT-gels fractured. The SDS-gels fractured at a much lower stress but at a much higher strain than those at which the control and DTT-gels fractured. It is clear that the DTT-gels were more brittle than the control gels, whereas the SDS-gels were much more rubbery. Three of the six samples of SDS-gel did not fracture at the maximum applied strain of 1·6 (i.e. the Hencky strain at 80% compression of the sample was calculated to be 1·6; Hamann & Foegeding, 1994).

Statistical analysis showed that for fracture strain, the three gel systems were significantly different from each other ($P<0·0005$). For fracture stress, SDS-gels were significantly different from the control gels ($P<0·0005$) and the DTT-gels were also significantly different from the control gels at ($P<0·05$).

### *PAGE analyses of the heated WPC solutions*

It was necessary to obtain samples of the heated WPC solutions for PAGE analysis before they form strong gels (Havea et al. 1998); therefore, the WPC solutions for PAGE analyses were heated for only 10 min at 85 °C.

Both the alkaline-PAGE (Fig. 5a, lane 1) and SDS-PAGE (Fig. 5b, lane 1) patterns of the unheated control WPC solution showed the monomeric whey protein bands of β-lactoglobulin, α-lactalbumin and bovine serum albumin (BSA). There was disulphide material on top of the stacking gels that did no enter the SDS-gel, also reported elsewhere (Havea et al. 1998). The alkaline-PAGE patterns of the heated WPC solution (Fig. 5a, lane 2) showed that almost all the whey protein was at or near the top of both the resolving and the stacking gels in the PAGE pattern. Almost all of the bands seen in lane 1 had disappeared, indicating essentially complete denaturation and covalent aggregation of the native whey proteins. There were some smeary bands, which were probably non-native monomeric or dimeric proteins (Hong & Creamer, 2002). SDS-PAGE analysis (Fig. 5b, lane 2) showed that some material, corresponding to disulphide-linked proteins, remained on top of the stacking gel (Havea et al. 1998). Some of the material was SDS-monomeric bands, corresponding to both β-lactoglobulin and α-lactalbumin. These results (Fig. 5, lanes 1 and 2) confirmed that, when the WPC solution was heated, all the proteins were denatured and aggregated and that some of these were dissociable under SDS conditions (non-covalently bonded) whereas some were not (disulphide bonded).

When the WPC solution that contained SDS was heated and analysed using alkaline-PAGE (Fig. 5a, lane 3), much of the whey protein appeared to have migrated as a poorly defined series of monomer and dimer bands. Some material remained near the top of the stacking gel and corresponded to disulphide-linked protein aggregates (Havea et al. 1998). SDS-PAGE analysis of the same sample (Fig. 5b, lane 3) showed disulphide linked material that remained on top of the stacking gel, dimeric species, as well as the SDS-monomeric proteins. The monomer bands corresponded to the same bands shown in the unheated sample (compare lanes 3 and 1 in Fig. 5b). These results showed that heating the WPC solutions in the presence of SDS, disulphide bonded aggregates were formed. At this stage (10 min heating at 85 °C), small quantities of disulphide bonds had been formed, and much of the protein remained as SDS monomers.

When the WPC solution that contained DTT was heated and analysed using alkaline-PAGE (Fig. 5a, lane 4), no

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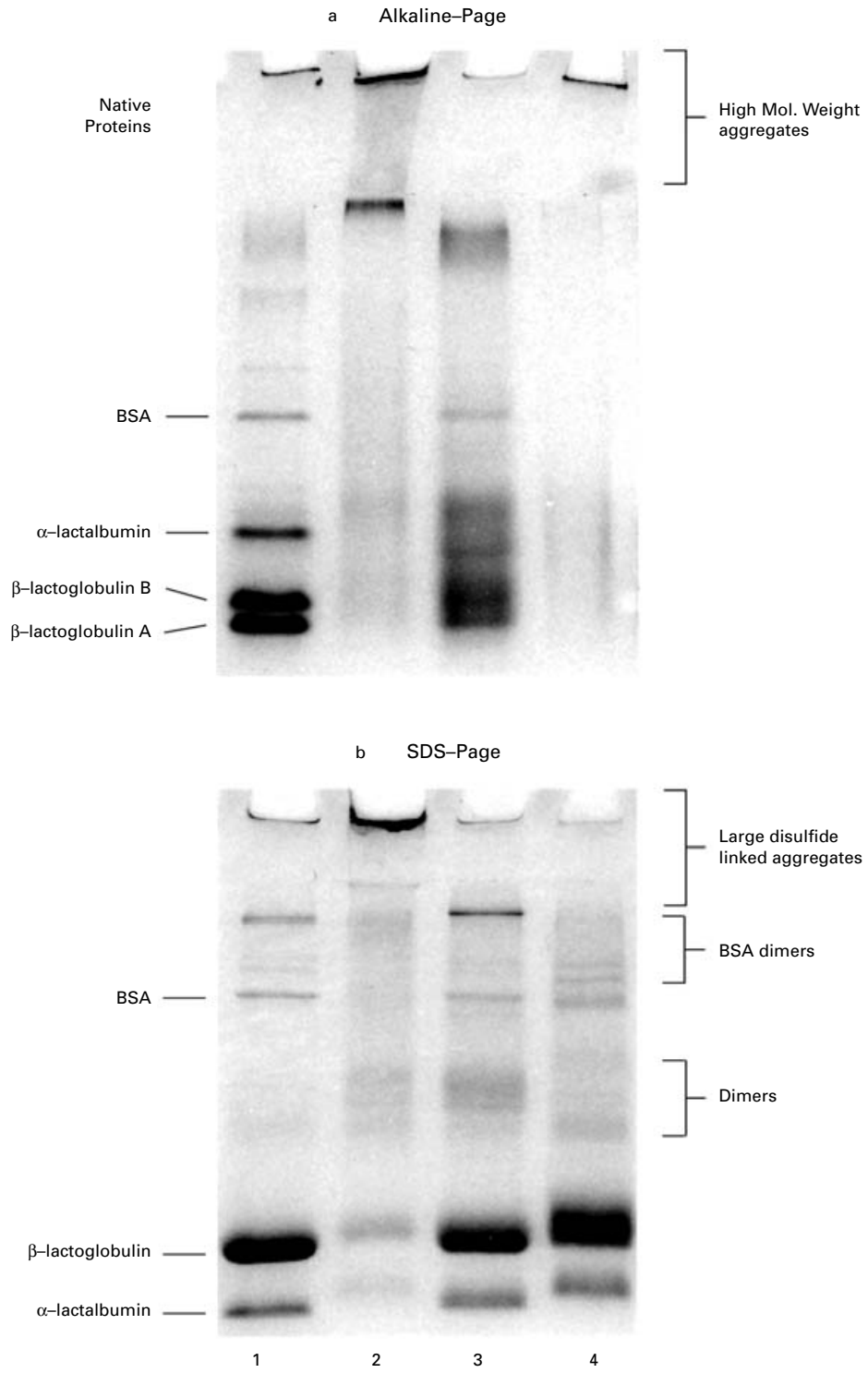


**Fig. 5.** (a) Alkaline-PAGE and (b) SDS-PAGE patterns of 150 g WPC/kg solutions, pH 6·9, heated at 85 °C for 10 min before analysis: lane 1, unheated control; lane 2, heated control; lane 3, WPC solution heated in the presence of 10 g SDS/kg; lane 4, WPC solution heated in the presence of 10 mM-DTT.

clear protein bands were observed on the gel, indicating complete denaturation of the whey proteins. When the same solution was analysed using SDS-PAGE, all the whey proteins were dissociated and migrated into the gel, forming bands corresponding to the SDS-monomeric proteins (Fig. 5b, lane 4). No material remained on top of the stacking gel, indicating an absence of disulphide-linked aggregates. These results showed that, upon heating the

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WPC solution containing DTT, all the whey proteins had been denatured and had formed aggregates. All of these aggregates were linked by non-covalent interactions and were dissociable under SDS conditions.

## Discussion

The interactions between the various proteins in WPC that result in a protein gel (which, in effect, means that there is a continuous protein phase that extends throughout the whole system) were significantly modified by introducing either SDS or DTT into the system, as shown by the results presented above.

These results give a new dimension to our understanding of the mechanisms of the heat-induced gelation of whey proteins. In this study, we modified the principal cross-linking mechanisms between the denaturing protein molecules during heating of the WPC solutions to primarily disulphide bonding (i.e. heating in the presence of SDS) or primarily non-covalent bonding (i.e. heating in the presence of DTT).

When SDS is added to a protein solution, the initial reaction is that the SDS binds strongly to the regions that are hydrophobic and carry a positive charge. At higher SDS : protein ratios, the binding becomes less specific and, finally, at an SDS : protein ratio (w/w) approaching 1·5, certain parts of the protein become nuclei for the formation of SDS micelles. At the concentrations used in the present study (10 g SDS/kg, 128 g protein/kg), the SDS would have been reversibly bound to most of the positively charged residues, and to some of the more specific hydrophobic regions, but, in essence, each protein entity would have acquired a significantly greater net negative charge. Thus β-lactoglobulin would have been wholly in the monomeric state and the ability of β-lactoglobulin or BSA and α-lactalbumin to form molten-globule-like adducts would have been lessened. Thus the inter-protein disulphide bond interchanges would have been slower, and the size of the aggregates would have been smaller. This is seen by comparison of lanes 3 and 2 in Fig. 5, which shows more material with higher mobilities and less material with lower mobilities (top of the PAGE pattern) in lane 3.

DTT is a powerful disulphide-bond reducing agent and the addition of a 10 mM concentration to a 150 g WPC/kg solution (128 g/kg protein) is likely to convert the disulphide bonds to thiol groups. The particle size evidence (Fig. 1c) and the rate of structure development (Fig. 2c) confirmed this. The SDS-PAGE analysis showed that the basic disulphide-bonded structures were primarily non-native monomers (lower mobility because of fewer disulphide bonds) and dimers. Thus, the DTT-gels were almost totally reliant on the hydrophobic effect mediated association of monomeric and dimeric whey protein species.

Disulphide-mediated polymerization of globular proteins is a well-documented phenomenon. Whey protein molecules are stabilized by intramolecular disulphide bonds. In addition, some (e.g. β-lactoglobulin and BSA) have one free thiol group. When the whey protein solutions are heated, inter- and intramolecular disulphide bonds are formed either by thiol/disulphide interchange or by thiol/thiol oxidation reactions (Dunkerley & Zadow, 1984; To et al. 1985; Monahan et al. 1995).

As mentioned earlier, the non-covalent interactions in heated protein solutions involve several distinct types, including hydrogen bonding, ion pairs, hydrophobic interactions and van der Waals' forces. Although these forces have been known for some time to be involved in the formation of heat-induced protein aggregates/gels, their respective roles remain unclear (Privalov & Gill, 1988; Dill, 1990; Matthews, 1995; Karplus, 1997; Jaenicke & Lilie, 2000). In our study, as we were able to take advantage of the fact that all these interactions are dissociable under SDS-PAGE conditions (Havea et al. 1998), we could prevent their formation in our heated WPC solutions. Under normal conditions, as in our control WPC solutions, both disulphide and non-covalent bonding occurred.

In a recent publication (Havea et al. 2002), we showed how two commercial WPC products exhibited different aggregation behaviours under the same heating conditions (120 g/kg, pH 6·9, 75 °C for up to 15 min). In the cheese WPC solutions, the whey proteins denatured at relatively fast rates, forming predominantly non-covalently linked aggregates that were particulate in structure. In the acid WPC solutions, the whey proteins denatured relatively slowly, giving rise to the formation of smaller aggregates that were linked, to a considerable degree, by disulphide bonding and formed a fine-stranded structure. The differences were attributed to the different calcium contents of the two WPCs. The high calcium content of the cheese WPC was considered to be responsible for mediating the formation of the non-covalent associations among the denatured proteins. The calcium probably lowered the net negative charge on the proteins, thus allowing them to form larger molten-globule-type aggregates more readily. It could also be possible that during heating calcium phosphate form precipitates together with proteins.

The results of the current study are consistent with the previously reported results (Havea et al. 2002); the three WPC solutions produced very different heat-induced gels. The SDS-gels, formed by disulphide bonding only, were translucent, had fine structure (Fig. 1b) and were formed relatively slowly during heating (Fig. 2b, Table 1). They were also softer and more rubbery than either the control gels or the DTT-gels (Fig. 4). In contrast, the DTT-gels, formed by non-covalent bonding only, were opaque, had a particulate structure (Fig. 1c) and were formed relatively rapidly during heating (Fig. 2c, Table 1). These gels were rigid and more brittle than the control gels or the SDS-gels (Figs 3 and 4). The control gels were formed by both disulphide and non-covalent bonding, and had characteristics that were intermediate between those of the SDS-gels and the DTT-gels (Figs 1–4). It appears that, the more the

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disulphide bonding dominates in the WPC gels, the more fine-stranded the gels were, a trend consistent with that observed by Havea et al. (2002).

It was interesting to note the different gel points of the heated WPC solutions. The DTT-gel started to form very early in the heating cycle, at 63 °C (Fig. 2c, Table 1). This temperature is around the thermal transition temperatures of BSA (64 °C) and α-lactalbumin (62 °C) (de Wit, 1984; Kinsella & Whitehead, 1989). However, it is unlikely that the changes in G′ values during gel formation (Fig. 2c) were due to the denaturation of these proteins alone. By the time the temperature reached 80 °C, G′ had reached a value >5000 Pa. The presence of DTT in these WPC solutions would have reduced many of the intramolecular disulphide bonds that maintained the globular structure of these molecules. Therefore, the protein molecules would have been partially denatured, possibly similar in structure to the molten globule state (Kuwajima, 1989), and held together by non-covalent interactions. As the heating progressed, the protein molecules would have unfolded readily, exposing the hidden hydrophobic residues and forming intermolecular linkages with each other. Because of the numerous reactive sites on the unfolded molecules, and the lack of restriction of movement by disulphide bonds, the chance of forming intermolecular linkages upon collision with another molecule would have been high. Consequently, gel formation occurred quickly, and strong gel networks were formed (Fig. 2c).

The WPC solutions heated in the presence of SDS had a delayed gel point (Fig. 2b, Table 1), and produced a weak gel at the end of the heating cycle. Although the proteins were denatured almost completely after 10 min of heating at 85 °C (Fig. 5a), it took much longer for the solutions to form a gel network. In the absence of non-covalent association, and because of the limited number of reactive sites (-SH and -S–S-), the interactions between the denatured proteins would have been relatively slow. Protein molecules would have had to collide in a certain way in order to effectively form a disulphide bond between molecules. Furthermore, the presence of SDS effectively charged the proteins negatively. The negative net charges of the proteins resulted in repulsive forces between them, hence slowing down the formation of a gel network (Fig. 2b). In preliminary work, we heated 120 g WPC/kg solution in the presence of SDS, but a gel did not form after heating for an hour at 85 °C (results not shown). Because of the limited linkages between the protein molecules, the heat-induced gels formed were weak and more rubbery.

These results demonstrate that the non-covalent interactions between the protein molecules in heated WPC solutions are very important. It was interesting to note that the characteristics of the DTT-gels (formed by non-covalent bonding) were closer to the characteristics of the control gels (Figs 1–4), than to those of the SDS-gels. This suggested that the heat-induced gelation of WPC solutions is probably dominated by the non-covalent bonding. In our previous work (Havea et al. 2000), we proposed a possible mechanism for the polymerization of whey proteins based on electrophoretic examination of intermediate protein aggregates formed during heating. This mechanism, as well as many others (e.g. Mulvihill & Kinsella, 1987; McSwiney et al. 1994b; Gezimati et al. 1997), was based mainly on the formation of disulphide bonds between protein molecules during heating, with little or no emphasis on the importance of the non-covalent bonding in the heat-induced aggregation/gelation of globular proteins. On the basis of the results presented in this current work, we have reason to suggest that heat-induced gelation of whey proteins under most conditions is dominated by non-covalent associations.

## Conclusions

The heat-induced gelation of WPC solutions is dominated by the non-covalent bonding between the denatured protein molecules. The disulphide bonding appeared to give the rubbery characteristics of the WPC gels whereas the non-covalent bonding their rigidity and the brittle characteristics. The non-covalent bonding gave particulate structure whereas the disulphide bonding gave fine-stranded structure. For the WPC to produce a desired type of gel, it is important to control the types of bonding that dominate the gels.

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