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The unexpected behaviour of 1,5-glucono-δ-lactone-induced myosin gels upon dialysis

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Dialysis of 1,5-glucono-δ-lactone-induced myofibrillar protein and myosin gels unexpectedly gave rise to stronger gels rather than a reversion to the original ungelled state. Dialysis of myosin also resulted in the formation of gels.

INTRODUCTION

Myosin and some of its fragments have shown reversible gel characteristics under certain conditions of pH, ionic strength and temperature. Wright and Wilding (1984) found that the thermal denaturation of the hinge region of rabbit myosin at pH 6.0 and ionic strength of 1.0 was reversible. When Samejima *et al.* (1976, 1981) studied the thermal denaturation of myosin rod, reversibility of thermal denaturation was also reported. Rogers *et al.* (1987) reported that the thermal unfolding of myosin rod obtained from four different organisms was highly reversible in the temperature range 0–80°C. Arteaga and Nakai (1992) observed full reversibility of the thermal denaturation of turkey breast myosin when heated for up to 30 min at 40°C, for 5 min at 50°C and when stored for 24 h at 4°C.

By analogy with gels induced thermally, the denaturation and formation of gels through pH alteration would be expected to be reversible. The aim of this study was to investigate the reversibility or otherwise of 1,5-glucono-δ-lactone (GdL)-induced myofibrillar protein and myosin gels using dialysis as a means of slow pH increase.

MATERIALS AND METHODS

Materials

Bovine *M. cutaneus trunci* was obtained from local freezing works within 1 h of slaughter. Water had been passed through a Milli-Q reagent water system (Millipore Corporation, Bedford, Massachussetts, USA) unless stated otherwise. All chemicals used were at least of analytical reagent grade.

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Preparation of myofibrillar protein

Meat was trimmed of excess visible fat and connective tissue, cut into approximately 3 cm cubes and stored at 4°C overnight. Ice and tap water was added to the cubed meat (1:1:1, w/w/w) in a Jeffco Wet Disintegrator (model 291, Jeffress Bros Ltd, Brisbane, Australia). The mixture was stirred for 5 min. The meat slurry was poured into a motorized mixer with a further addition of iced tap water (2:3, w/w). The mixture was stirred for 10 min during which time collagen fibres were removed with a spoon. The meat slurry was strained through a 3 mm stainless steel mesh to remove residual collagen. Discs were removed from a Westfalia Separator AG (model LWA 205, Westfalia Oelde, Germany) which was operated at 6300 ***g***. The slurry was added slowly to the separator until a maximum amount of myofibrillar protein, which collected on the inner surface of the bowl, was retained. Liquor was collected and run once through the separator. The myofibrillar protein was mixed in a Kenwood Chef Cakemixer (model A703C, Australia) to achieve homogeneity. The protein was then vacuum-packaged and heat-sealed using a chamber type vacuum packaging machine. It was stored as discs, less than 5 mm thick, at −20°C in PVDC nylon polyethylene polymer bags with low oxygen permeability and low moisture permeability. Myofibrillar protein was used within 5 months of preparation.

Myosin preparation

The method of preparation of myosin was based on the procedure described by Dudziak & Foegeding (1988) with the following modifications:

- meat was minced in a Sunbeam Kitchen Whizz instead of being passed through a grinder;
- after dilution to an ionic strength of 0.03, the precipitate was allowed to settle overnight. Liquid was

then carefully siphoned from the settled precipitate prior to centrifugation;

- the protein solution was not dialysed in the final stages of the preparation, but was diluted 10-fold with water. The suspension obtained was centrifuged at 10 800 *g* for 30 min and a crude myosin preparation was obtained. The protein was stored in a plastic container at 4°C for up to 8 days.

Myosin preparation was undertaken at 4°C. Sodium azide (0.1 g kg^{-1}) was added to the protein preparation to slow microbial degradation.

Gel preparation

Myofibrillar gel preparation

Myofibrillar protein (100 g, 800 g kg^{-1} moisture) was thawed for 30 min at room temperature (approximately 20°C) and then ground in a mortar and pestle for less than 30 s. Water (10 ml) or a solution of sodium chloride (NaCl; 2.50 g) and/or tetrasodium pyrophosphate (TSPP; 0.30 g) in water (10 ml) was added to the myofibrillar protein sample approximately 1 h 20 min after removal of the protein from the freezer. The mixture was stirred for 30 s. Water (20 ml) or a solution of GdL (2.00 g) in water (20 ml) was added to the protein mixture 5 min later. The mixture was stirred for 30 s.

In one set of experiments, gels were formed in dialysis tubing (viscose cellulose, molecular weight cut-off approximately 12 000–14 000, Union Carbide 453105) as cylinders (15 mm diameter, approximately 50 mm length) and the ends were clipped with plastic dialysis clips.

In a second set of experiments, to achieve more uniform dialysis, samples were placed in vertically suspended dialysis tubing (approximately 120 mm length, 23 mm width) between two parallel perspex plates which were separated by a 1 mm gap. The tubing was clipped at the base using a metal bulldog clip. The suspensions were forced into the tubing to fill the gap and the gelation allowed to take place at 4°C over 24 h. Prior to removal from the plates, the tubing was sealed at the top with a metal bulldog clip.

Myosin gel preparation

A sample of 20 g $litre^{-1}$ myosin suspension (4.50 g) was measured into a plastic beaker. Where NaCl (0.110 g) and TSPP (0.011 g) were added, these compounds were added directly to the myosin and well stirred. Where GdL (0.015, 0.031 g) was added to the myosin, it was added immediately after mixing in the NaCl and/or TSPP, and was well mixed. These weights give GdL concentrations of 3.4 and 7.0 g kg^{-1} in the water phase of the myosin system. In all subsequent references to weight per weight concentrations, the concentrations are based on a weight per weight ratio with the water phase of the system.

Gels were formed in dialysis tubing as cylinders or 1 mm strips as detailed for myofibrillar protein gel preparation. However, the length of the myosin strips was 60 mm, not 120 mm.

Dialysis

As a dialysis control, gelled myosin samples which had formed in dialysis tubing (15 mm diameter, approximately 50 mm length) were removed from the tubing and placed in water (1.0 litre).

Samples (15 mm diameter, approximately 50 mm length) of gelled myofibrillar protein or myosin in dialysis tubing were suspended in water (1.0 litre) or water whose pH had been adjusted with 0.1 M sodium hydroxide or 0.1 M sodium hydrogen carbonate. The dialysis liquid was changed at least once during the dialysis process. Dialysis was carried out for at least 24 h at 4.0°C with slow stirring provided by a magnetic stirrer.

To achieve uniform dialysis, myosin and myofibrillar protein were gelled as flat strips (1 mm thickness, 120 or 60 mm length and 23 mm width). The ends of the strips secured by bulldog clips rested on the bottom of the dialysis vessels instead of being suspended. These samples were carefully rocked, not stirred.

Sodium azide (0.1 g kg^{-1}) was added to the dialysis liquid to slow microbial degradation of the sample.

Tensile tests

Tensile tests were undertaken on the Instron Universal Testing Machine (model 4502, Instron Ltd., High Wycombe, UK). Myosin and myofibrillar protein samples dialysed as 1 mm thin strips and their undialysed counterparts were cut into samples of 23.0 mm length and 7.5 mm width. The samples were suspended from bulldog clips attached to horizontal probes and tensile tests were undertaken, in triplicate, by extension of the sample at a rate of 25.4 mm min^{-1}. A 10 N load cell was used and extension of the sample was taken to destruction. Force and displacement at the point of destruction were recorded. Many of the samples, in particular the undialysed samples, were too fragile to be suspended from the clips and no data could be obtained.

Transmission electron microscopy (TEM)

Transmission electron microscopy was undertaken by the Electron Microscopy Laboratory, Hort + Research, Palmerston North, New Zealand.

Small pieces of myofibrillar gels were fixed in a primary fixative of 30 g kg^{-1} glutaraldehyde and 20 g kg^{-1} formaldehyde in 0.1 M phosphate (Na_2HPO_4–KH_2PO_4) buffer (pH 7.2) for 2 h at room temperature. The pieces of gels were then washed three times in the buffer at room temperature. The gel pieces were placed in a secondary fixative of 10 g kg^{-1} osmium tetroxide in phosphate buffer (pH 7.2) for 1 h at room temperature. Dehydration was undertaken using a graded acetone series (0.25, 0.50, 0.75, 0.95, 2 × 1.0 litre $litre^{-1}$) in the

buffer. Infiltration of resin into the gels was undertaken in an acetone:resin (50:50) mixture (Polarbed 812 epoxy resin). The mixture was stirred overnight at room temperature. The pieces of gels were placed in resin and stirred for 7 h at room temperature. The gel fragments were embedded in fresh resin in a silicone rubber mould and cured at 60°C for 48 h.

Sections of 90 nm (pale gold interference colour) were cut from the trimmed blocks using a diamond knife and a Reichert Ultracut E ultramicrotome. Sections were grid mounted and double-stained using saturated uranyl acetate in 0.50 litre^{-1} ethanol, followed by lead citrate (Venable & Coggleshall, 1965). Sections were studied using a Philips 201c Transmission Electron Microscope.

Analytical methods

Analysis of myofibrillar protein

Moisture, fat and protein content was determined by the Food Technology Research Centre, Palmerston North and M.I.R.I.N.Z., Hamilton. Collagen was determined by M.I.R.I.N.Z., Hamilton and the AgResearch Analytical Services Laboratory, Palmerston North. The hydroxyproline content was measured after acid hydrolysis in 6 M concentrated hydrochloric acid and a conversion factor of 7.14 was assumed to calculate the collagen concentration.

Analysis of myosin preparations

Total protein content was determined using the biuret procedure described by Gornall *et al.* (1949). Moisture content was determined according to AOAC (1990).

Myosin sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

The procedures for preparation of SDS–PAGE gels and sample preparation were adapted from the method described by Locker & Wild (1984). A Hoefer Mighty Small II Slab Gel Electrophoresis Unit (SE 250, Hoefer Scientific Instruments, San Francisco, USA) was used.

A 100 g litre^{-1} ammonium persulfate solution was used. A 45 g litre^{-1} acrylamide (100:1) stacking gel

Table 1. Results of tensile tests of GdL-induced myosin gels measured at the point of destruction

Additives (g kg^{-1})	Average force (N)	Standard deviation of force (N)	Average displacement (mm, average)	Standard deviation of displacement (mm)	Final pH
Not dialysed[a]					
7.0 GdL + 25.0 NaCl	0.046	0.011	26.3	5.6	
7.0 GdL + 25.0 NaCl + 2.5 TSPP	0.079	0.011	34.4	2.7	
Dialysed					
—	0.083	0.006	21.2	2.9	5.7
3.4 GdL	0.038	0.008	17.8	8.0	
7.0 GdL	0.097	0.021	39.0	7.0	5.7
25.0 NaCl	0.138	0.043	15.5	5.7	
7.0 GdL + 25.0 NaCl	0.100	0.012	25.5	4.0	4.9
2.5 TSPP	0.053	0.015	15.6	0.4	
25.0 NaCl + 2.5 TSPP	0.089	0.020	14.0	1.2	
7.0 GdL + 25.0 NaCl + 2.5 TSPP	0.164	0.024	34.6	7.2	

[a]Many of the undialysed samples were too fragile for testing and only those reported were measurable.

Table 2. Tensile test of dialysed myofibrillar gels measured at the point of destruction

Additives (g)	Average force (N)	Standard deviation of force (N)	Average displacement (mm)	Standard deviation of displacement (mm)
2.0 GdL	0.153	0.018	17.6	4.0
2.0 GdL + 2.5 NaCl	0.106	0.019	5.6	1.2

Note: undialysed gels were too fragile for testing.

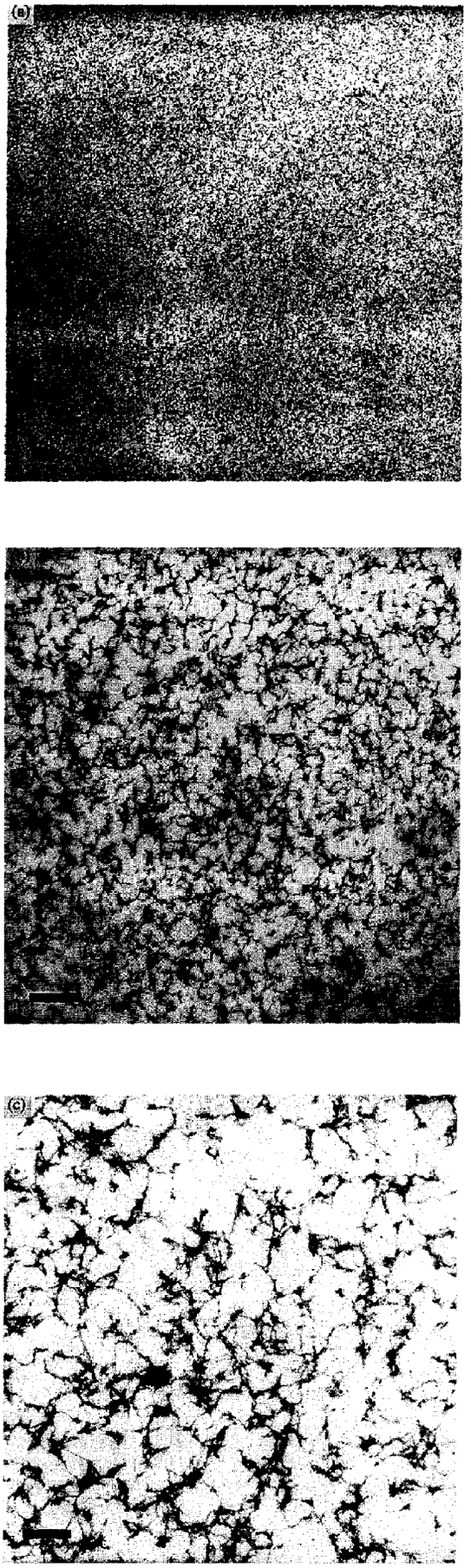

Fig. 1. Transmission electron micrographs of myosin gels with 7.0 g kg^{-1} GdL, after dialysis (pH 5.7). The bars represent (a) 1.0 μm, (b) 0.5 μm and (c) 0.2 μm.

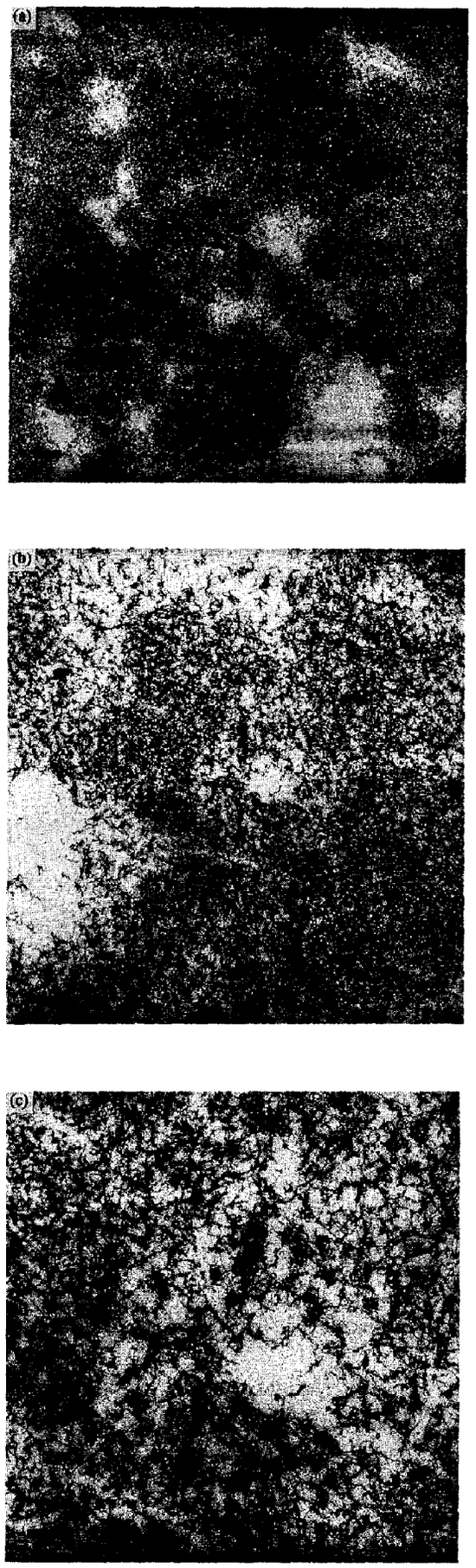

Fig. 2. Transmission electron micrographs of myosin after dialysis (pH 5.7). The bars represent (a) 1.0 μm, (b) 0.5 μm and (c) 0.2 μm.

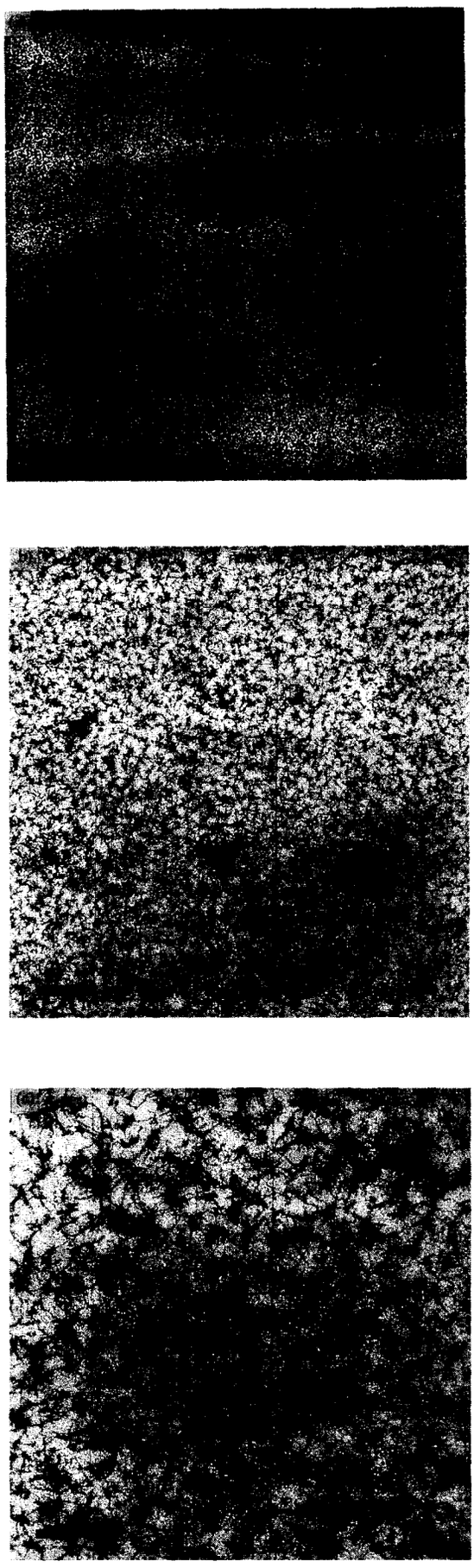

Fig. 3. Transmission electron micrographs of myosin with 7.0 g kg^{-1} GdL and 25.0 g kg^{-1} NaCl, after dialysis (pH 4.9). The bars represent (a) 1.0 μm, (b) 0.5 μm and (c) 0.2 μm.

Fig. 4. Transmission electron micrographs of myosin with GdL (pH 4.3). The bars represent (a) 1.0 μm, (b) 0.5 μm and (c) 0.2 μm.

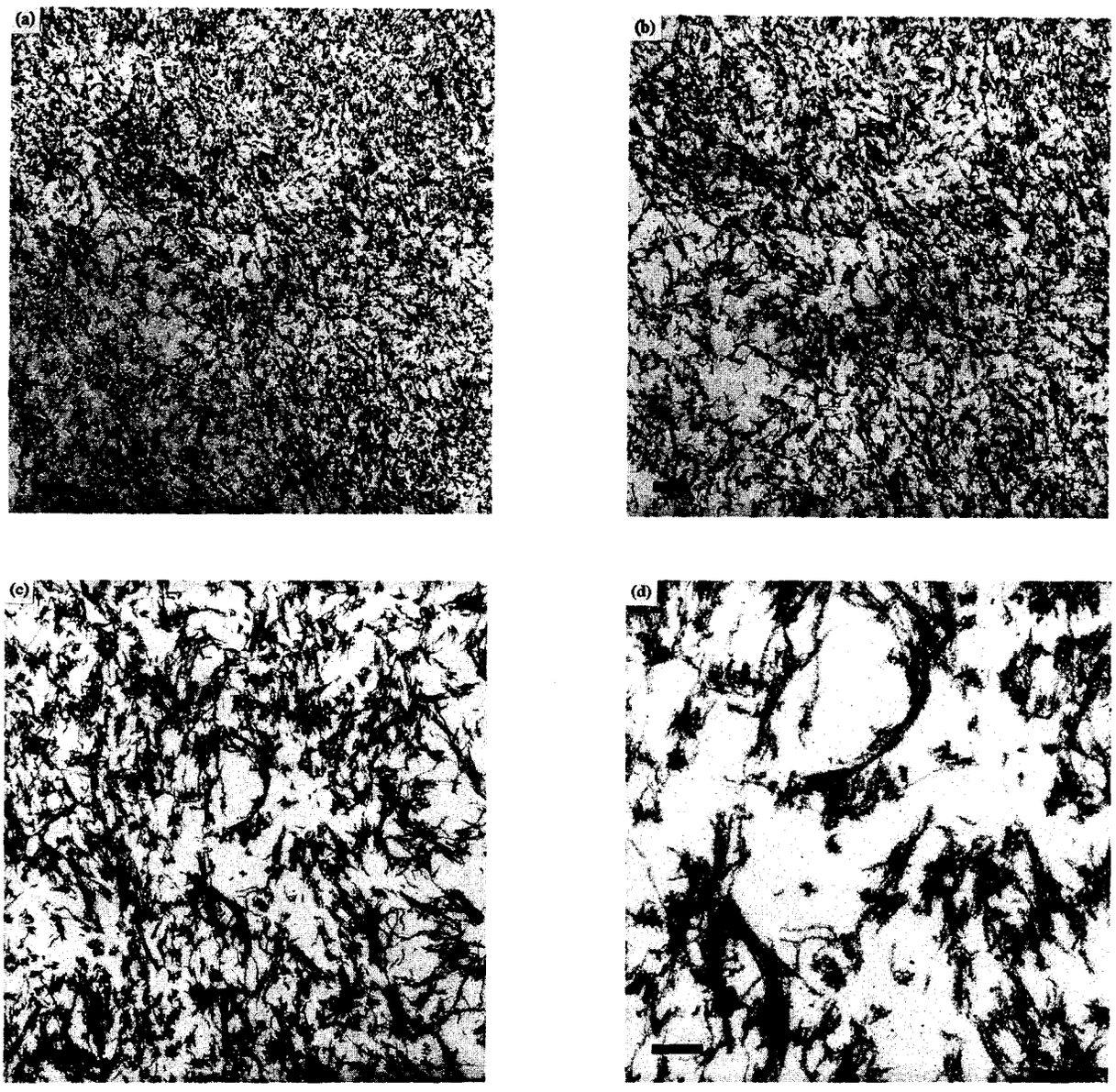

Fig. 5. Transmission electron micrographs of myosin (pH 6.8). The bars represent (a) 1.0 μm, (b) 0.5 μm, (c) 0.5 μm and (d) 0.2 μm.

system was used of the same proportions as the casting gel, but with a Tris–glycine solution of pH 6.8. Myosin samples were diluted to concentrations of 0.40 g litre^{-1} myosin with water. High molecular weight standards were prepared in the same way as the myosin samples. Gels were run for 45–60 min at 20 mA (approximately 150 V) per gel using a 500/200 V power supply.

Electrophoresis gels were scanned on a LKB Produker AB Ultrascan XL Laser Densitometer (Bromma Sweden).

Measurement of pH

Direct pH measurements were taken upon completion of tests, at 20°C using a standard pH meter (digital ionalyser, model 701, Orion Research Inc., USA) equipped with a glass electrode. Measurements of protein sample pH were calculated from the average of four replicates.

RESULTS

Analysis of protein preparations

The collagen content of the recovered myofibrillar protein was 0.86% (standard deviation 0.34%) of the wet weight and the fat content 0.27% (standard deviation 0.04%) of the wet weight. The moisture content was 82.0% (standard deviation 0.8%) and protein content was 17.8% (standard deviation 0.2%) of the wet weight.

The purity of the myosin preparations was 85–95% with an average of 91% by densitometry of the SDS–PAGE gels.

Dialysis of myosin

Control samples of myosin which had gelled with added

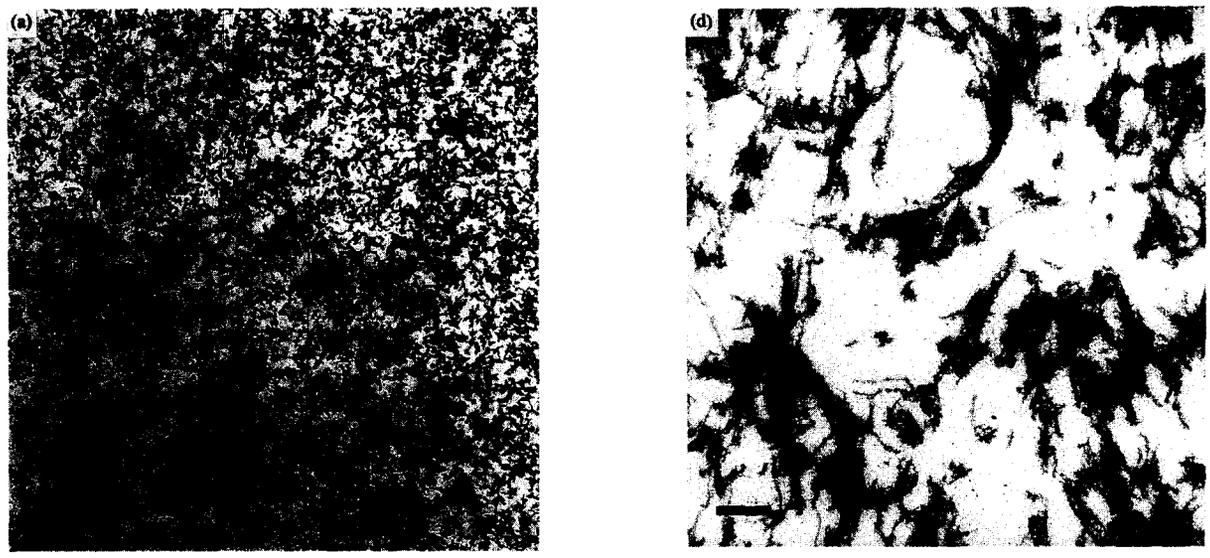

Fig. 6. Transmission electron micrographs of myosin with NaCl and GdL added (pH 4.0). The bars represent (a) 1.0 μm and (b) 0.2 μm.

7.0 g kg^{-1} GdL were placed in water. After several hours, material at the surfaces of the sample had dispersed as fibrils into the water. The gel had completely disintegrated after 8 h in the absence of the restraining dialysis tubing.

In contrast, after 24 h dialysis, rubbery, translucent layers, 2–3 mm thick, had formed at the surface of the myosin cylinders. However, the appearance of the central portion was unchanged from that of the myosin sample prior to dialysis. Extending the dialysis time to 72 h did not change either the thickness of the rubbery layer nor the appearance of the central portion. In order to overcome this lack of uniformity in the gels formed upon dialysis, myosin was gelled as 1 mm thick flat strips and dialysed.

Myosin strips with added 7.0 g kg^{-1} GdL were dialysed in water with an initial pH of 10.0 (adjusted with 0.1 M sodium hydroxide). The final pH of the water was 9.1. Visually, the formed gels did not appear different from gels that had formed upon dialysis in water and the final gel pH value was 5.7.

The results of tensile tests of myosin samples with added combinations of GdL, NaCl and TSPP, which had been allowed to stand for 24 h in dialysis tubing, and then dialysed in water, are presented in Table 1. Non-dialysed controls were too fragile for testing with the exception of the samples with 7.0 g kg^{-1} GdL plus 25.0 g kg^{-1} NaCl and 7.0 g kg^{-1} GdL, 25.0 g kg^{-1} NaCl plus 2.5 g kg^{-1} TSPP (Table 1). Significant increases in gel strength were observed in these samples after dialysis. Prior to dialysis the gels appeared opaque or translucent and white, with the exception that the addition of 25 g kg^{-1} NaCl formed colourless transparent solutions. After 24 h dialysis all gels appeared transparent and colourless.

Myosin alone formed a stronger gel after 24 h dialysis in water than if allowed to stand at 4°C for the same time (Table 1). The pH of myosin decreased from 7.0 prior to dialysis, to 5.7 after dialysis.

Figures 1–3 show TEM micrographs of myosin with 7.0 g kg^{-1} GdL, myosin alone and myosin with 7.0 g kg^{-1} GdL and 25.0 g kg^{-1} NaCl, respectively, each dialysed in water. The networks observed were more uniform in appearance and were finer and denser than networks which had not been dialysed (Figs 4–6).

Dialysis of myofibrillar proteins

In cylindrical samples of dialysed myofibrillar protein gels formed with GdL, differences were only observed at the surface of the gel in contact with the dialysis tubing, which became white and rubbery and had a pH of 5.6. The pH of the samples in the unchanged interior was 4.0, the same as the undialysed sample.

Cylindrical gels of myofibrillar protein with TSPP, NaCl and GdL dialysed in water, resulted in a grey, crumbly surface, which was not sticky and had a pH of 4.6, not significantly different from the control gel and the centre of the dialysed gel which both had pH of 4.5. An unpleasant fish-like odour was also noted. Dialysis in water maintained throughout at pH 6.0 with 0.1 M sodium hydroxide, resulted in a similar gel to that dialysed in water. The final gel pH was 4.5.

Myofibrillar protein gelled as cylinders with GdL and then dialysed in water with an initial pH of 8.0 (adjusted with 0.1 M sodium hydrogen carbonate addition and changed after 22 h) resulted in a gel with a firmer, white surface which was not sticky. The interior of the sample was sticky, pink and glassy, similar to the sample prior to dialysis. The pH at the core of the sample was 4.0 while the surface was 4.7. The sample which had not been dialysed had a pH of 4.5.

In contrast to myosin which changed in character on dialysis, the dialysis of 1 mm thick strips of myofibrillar

protein without added GdL did not result in an observable difference in physical appearance. The addition of NaCl to myofibrillar protein resulted in a pink, sticky sample which could not be handled without breaking. After dialysis, the gel could be handled, but could not be suspended by the clip for tensile testing without breaking under the force of the clip.

Dialysis of myofibrillar protein with added GdL and GdL with NaCl resulted in 'strong' white gels which could be tested using tensile force to destruction. The undialysed samples could be suspended, but were very fragile and disintegrated prior to the application of force. Results of the two samples tested are given in Table 2.

DISCUSSION

The non-uniformity of dialysed gelled myosin cylinders indicated that the outer surface was virtually impermeable to diffusion of ions and gluconic acid molecules. This phenomenon appeared to be analogous to that of case hardening in tanning. On changing the samples from a cylindrical shape to 1 mm thick strips the dialysed gels then appeared to be uniform throughout.

Gelled myosin samples which were not confined in dialysis tubing, when placed in quiescent water, disintegrated into fine fibrous particles within 8 h. These results indicated that the rate of pH change or ion diffusion was important in determining the reversion or otherwise of the GdL-induced gel. A slow rate of gluconic acid diffusion, imposed by the barrier of dialysis tubing, resulted in a stronger gel which was dense to the point of being effectively impermeable. A faster rate of pH change (that is, gluconic acid diffusion out of the gel) resulted in reversion to a non-gel form.

A straightforward explanation for the observed increase in gel strength of acid-induced myofibrillar protein and myosin gels upon dialysis is that the isoelectric point (5.4, Szent-Györgyi, 1951) is approached as the pH increases. The net charge on the protein thus changes, and repulsive forces between the protein molecules are minimized, resulting in a firmer gel. However, this explanation cannot readily account for the observation that a relatively fast rate of pH increase resulted in gel disintegration, whereas a slow rate of pH increase (on dialysis) gave a stronger gel.

An explanation can be based on studies of myosin gelation at different heating rates. Foegeding *et al.* (1986) observed that 6 mg ml^{-1} myosin heated to 70°C at 12°C h^{-1} produced more rigid gels than at 50°C h^{-1}. Egelandsdal *et al.* (1986) found that decreasing the heating rate from 150 to 6°C min^{-1} had a large positive effect on the storage modulus of myosin gels at 10 mg ml^{-1}. These results indicate that the slower rate of heat-induced denaturation favours ordered rather than random protein–protein interactions, with the former resulting in stronger gels. By analogy, the slower pH increase obtained in dialysis would allow slow rate of denaturation and aggregation of the protein molecules, favouring the ordered protein–protein interactions required for strong gel formation (Ferry, 1948; Hermansson, 1979).

The appearance of the dialysed gels in the micrographs of myosin, myosin with 7.0 g kg^{-1} GdL and myosin with NaCl and 7.0 g kg^{-1} GdL (Figs 1–3) showed a fine network without the appearance of aggregates or filaments in sharp contrast to the samples which had not been dialysed (Figs 4–6). The gel network may be said to have become denser. These results support the suggestion that there is a change in the nature of the network of myosin after dialysis.

Similar explanations can be advanced for the results observed for the myofibrillar protein system.

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