HEAT-GELATION OF MYOSIN : INFLUENCE OF PURIFICATION, MUSCLE TYPE AND IONIC STRENGTH

C. BOYER, A. OUALI AND J. CULIOLI

Station de Recherches sur la Viande - INRA Theix, 63122 St Genès-Champanelle

SUMMARY : Heat-gelation of myofibrillar proteins and myosins isolated from rabbit fast-twitch *Psoas Major* (PM) and slow-twitch *Semimembranosus proprius* (SMp) muscles was studied at different stages of purification. Gelation was induced by heating at a 0.5°C/min rate in the 30-80°C temperature range, at pH 6.0 and 2 ionic strengths (KCl 0.2 or 0.6M). At high ionic strength, heat-gelation of myofibrillar proteins occurred in two steps, with first, a rigidity peak at 48°C and 53°C for PM and SMp muscles, respectively, and then, an increase up to 80°C. The purification of myosin induced the loss of the peak and an increase in the final rigidity modulus. Both myofibrillar proteins and myosin isolated from SMp muscle exhibited a higher heat-stability and a lower gelling strength, than those from PM muscle. Heating of myosin dispersion in 0.2M KCl gave weak gels. In contrast, dialysis against 0.2M KCl of proteins, previously dissociated in 0.6M KCl, induced gel formation at low temperature and a 2 fold more rigid gels after heating than those obtained at 0.6M KCl. Differences in the heat-gelling behaviour were related to the aggregated or filamentous structure of the gels.

INTRODUCTION : Heat-gelation properties of myofibrillar proteins especially myosin, have been extensively investigated over the last decade. Several reports have emphasized the effect of the myosin type and its degree of purification on heat-gelling properties of this major muscle protein (FRETHEIM *et al.*, 1986; SANO *et al.*, 1989). However, only few investigations have been carried out on perfectly homogeneous fibre type muscles and on highly purified myosin isoforms. Moreover, contradictory results were obtained about ionic strength effects and molecular mechanisms involved in the gelation process (ISHIOROSHI, 1979, 1983 ; EGELANDSDAL *et al.*, 1986 ; SANO *et al.*, 1990). The objectives of this work was (i) to analyze the heat-gelling behaviour of proteins from extreme homogeneous muscle types (Type IIB and type I), (ii) to determine the incidence of the degree of myosin purification (in particular C protein elimination), and (iii) to clarify the ionic strength effects.

MATERIALS AND METHODS :

1 - Extraction and purification of proteins : Protein extracts were prepared from rabbit *Psoas major* (PM) and *Semimembranosus proprius* (SMp) muscles, classified as type IIB and I respectively (DUFOUR *et al.*, 1989). The muscles were excised just after slaughter. One part of each was immediately used for myofibrils preparation, whereas the second was kept in ice for 1h and used for myosin extraction. Myofibrils (fraction 1) were extracted according to OUALI and TALMANT (1990) and the final pellet resuspended in 40mM potassium phosphate buffer pH 6.0, 0.6M KCl (buffer (D)). Myosin was purified according to the method of OFFER *et al.* (1973) with minor modifications. Muscle homogenised in 4 volumes of buffer (A) (0.3M KCl, 0.15M potassium phosphate, 10mM tetra-sodium diphosphate pH 6.4) was kept at 0°C under stirring for 15 min. After centrifugation (15 min. at 30000xg), solubilised proteins were precipitated by addition of 14 volumes of cold distilled water and cleared of the sarcoplasmic fraction. The pellet was then washed with 20 fold diluted buffer (A) (fraction 2). This crude myosin extract was dissolved in buffer (B) (0.5M KCl, 0.05M potassium phosphate, 1mM EDTA, 10 mM tetra-sodium diphosphate, pH 7.3) and actin further eliminated by addition of ammonium sulphate to a final concentration of 1.4M and 1.3M for PM and SMp muscle myosins, respectively. Myosin was then separated from F protein by ammonium sulphate precipitation (1.73M). Finally, myosin, C protein and some trapped F protein (fraction 3) were dissolved in buffer (C) (0.15M potassium phosphate, 10mM EDTA, 10mM tetra-sodium diphosphate, pH 8.0) and proteins were separated by chromatography on a DEAE Sephadex A50 gel. Myosin was eluted by a 0.6M KCl solution. Pure myosin (fraction 4) precipitated by a 48h dialysis against a 50mM KCl solution and then centrifuged for 30 min. at 30000xg. Fractions 2, 3 and 4 were dissolved in buffer (D) and equilibrated to the final ionic strengths by dialysis against the same buffer containing 0.2M or 0.6M KCl.

Protein contents were determined by the Biuret method (GORNALL *et al*, 1949) and composition of each fraction analysed by SDS-PAGE on 12.5% slab gels according to LAEMMLI (1970).

2 - Rheological measurements : Heat-gelation of myofibrillar proteins and myosin was studied using a Carri-Med CSL 100 rheometer in oscillatory conditions. Protein samples were heated between the 2 parallel plates of the rheometer (D = 4 cm, gap = 1 mm) from 30°C to 80°C at a rate of 0.5°C/min.. Rheological assays were performed with a maximal deformation of 2%. Storage (G') and loss (G") moduli as well as loss tangent (δ) were determined after every 0.5°C increment.

3 - Scanning Electron Microscopy (SEM) : Myosin gels were obtained by gradual heating at a 0.5°C/min rate from 30°C up to 70°C. Gel pieces were immersed in 2.5% glutaraldehyde in buffer (D) (0.2M or 0.6M KCl) for 1h at 4°C. A post-fixation was performed in 1% osmium tetroxyde for 1h. Fixed specimens were dipped in buffer (D) and dehydrated in graded ethanol solutions (70, 95 and 100%). Samples were dried using carbon dioxide critical point drying method, coated with a 20 nm gold layer in a vacuum evaporator and, finally, observed with a 505 Philips SEM at an accelerating voltage of 25 or 30 KV.

RESULTS AND DISCUSSION :

1 - Protein identification : Electrophoretic patterns of protein fractions (figure 1) show that the chromatography step on a DEAE Sephadex A50 column is essential to obtain myosin cleared of C protein (lanes 3 and 4). Moreover, the presence of only fast light chains (LC1f, LC2f and LC3f) (lane 4) and slow light chains (LC1s and LC2s) (lane 5) in the case of PM and SMp myosin respectively attests of the homogen fibre content of these muscles a finding comforting earlier ones based on pattern analysis of native myosin isoform (DUFOUR *et al.*, 1989).

2 - Heat-gelation of proteins at 0.6M KCl :

a) Purification effects : heat-gelation of myofibrillar fractions is a two-step process (figures 2 and 3). Regarding PM proteins (figure 2), a first transition (T1) is observed near 40°C : rigidity moduli (G' and G'') increase simultaneously to reach a maximal value at 48°C (T2). At this temperature, the loss tangent δ is still higher than 10° indicative of the high viscosity of the gels. Then, G' and G" decrease rapidly until 51-52°C (T3). After T3, G' increases again while G" remains constant. This phenomenon induces a steep decrease of δ which reaches a low value (4°) characteristic of very elastic gel. EGELANDSDAL and MITCHELL (1987) described a similar behaviour of bovine myofibrils from fast-twitch skeletal muscle, with however, slightly higher transition temperatures. This could be explained by the fact that bovine muscles are of an intermediate type. Indeed, red SMp proteins exhibit a higher heat-stability as shown by the higher transition temperatures (T1, T2, T3), of 46, 53 and 56,5°C respectively (figure 3). The successive purification steps lead, on the one hand, to a modification of the rheogram profiles, in particular to a progressive decrease of the first rigidity peak which disappears after actin elimination (figure 2 and 3). This seems to confirm the observations of SANO *et al.* (1988, 1989) realised, at low ionic strength, on natural actomyosin and myosin from carp. On the other hand, the purification induced a 1,5 to 7,5 fold increase in the rigidity moduli at 80°C according to the muscle type, the purification step and the ionic strength (table 1). Such effects have also been observed by FRETHEIM *et al.* (1985).

b) Particular effect of C protein : This effect is strongly dependent on the muscle type. Indeed, C protein elimination leads to a decrease of about 1 to 2°C in the T1 value of PM myosins, whereas, for SMp myosin, this temperature increases by 5 to 6° (figure 2 and 3). Besides, C protein elimination induces an increase in the rigidity moduli at 80°C, a finding similar to that reported by YAMAMOTO *et al.* (1987) at low ionic strength.

c) Effects of muscle type on myosin gelation : As shown in table 1 and figure 4, proteins from SMp muscle exhibit a lower gelling ability than those from PM muscle, a result comforting earlier observations on chicken (ASGHAR *et al.*, 1984) and bovine (FRETHEIM *et al.*, 1986) myosins. Transition temperatures of myofibrillar fractions and myosins are muscle type dependent. As stressed by the greater T1 values (40 and 53°C for PM and SMp muscles, respectively), SMp myosins are clearly more thermostable. However, during SMp myosin gelation, a sligth increase in G' was observed between 40 and 50°C the extent of which seems to depend on the solubility of the initial unheated proteins (the more soluble the myosin, the higher the G' increase).This last observation confirms the hypothesis of CULIOLI *et al.* (1992) who related the heat stability properties of the two type of myosins to their solubility and their surface hydrophobicity. Indeed, as shown by OUALI *et al.* (1988) surface hydrophobicity of SMp myosin is higher than that of PM myosin, a difference which could explain the lower solubility of the former myosin type (NAKAI *et al.*, 1986).

3 - Ionic strength effects on heat-behaviour of proteins : Dispersion of myosin molecules in the buffer (D) (40mM potassium phosphate buffer) containing 0.2M KCl gave weak gels. In contrast, when the same myosin was first solubilised in buffer (D) containing 0.6M KCl and then adjusted to 0.2M KCl by dialysis against the above buffer, gelification occurs at temperature as low as 4°C (figure 5). This spontaneous gel exhibits a low rigidity value (figure 5) and a relatively high viscous component (δ value is close to 10°). Upon heating of these dialysed myosin a two step gelification is observed : G' and G" reaches a first maximum value at 49°C and 53°C for PM and SMp muscles respectively. Then, G' increases up to 80°C while G" modulus decreases and δ reaches a very low value (2°) characteristic of highly elastic

gels. Moreover, at highest temperature (80°C), as shown in table 1 and figure 5, the heat-gels from both muscles are 2 fold more rigid than those obtained at 0.6M KCl, which is in accordance with the results of ISHIOROSHI et al. (1979,1983), HERMANSSON et al. (1986), EGELANDSDAL *et al.* (1986) and YAMAMOTO *et al.* (1987). Aggregation of myosins into filamentous structures might account for the weak gels formed at low temperature. However, the interactions as well as the denaturation mechanisms of the different myosin subfragments upon heating of low or high ionic strength myosin preparations are however not elucidated and, in this respect, findings reported are still very controversal (SAMEJIMA *et al.*, 1983 ; EGELANDSDAL *et al.*, 1986 ; SANO *et al.*, 1990). Finally, it is worthy to note that a similar effect of the myosin type, previously described at high ionic strength, was observed at 0.2M KCl (table 1). However at 0.2M KCl the effect of purification is less pronounced and the influence of C protein elimination even desappears.

4 - **Structural analysis of protein gels :** SEM analysis of PM myosin gels in 0.2M KCl reveals a filamentous structure, already described by HERMANSSON *et al.* (1986), which evolves during the heating (figures 6). Thus, it appears that porosity is maximal after heating, whereas a denser and finer structure (with the smallest diameter of filament) is obtained at the temperature of the gelation peak. In contrast, structural analysis of SMp myosin gel in the same conditions reveals a more aggregated structure (figure 7). Examination of the gels from the two myosin types, at 0.6M KCl, does not reveal filamentous structures. Moreover, the differences observed in the rigidity moduli between PM and SMp myosin gels (figure 4) could be explained by their very different ultrastructures (figure 8 and 9). In PM myosin gel, structures appear finer and the network denser and more regular than in SMp myosin gel. Finally, as shown in figure 10, PM myofibrillar protein gel, at 0.6M KCl, exhibits a more globular structure than the corresponding myosin gel (figure 10). These results would suggest that as gel structures become finer, their rigidity modulus (G') increases.

REFERENCES :

ASGHAR A., MORITA J., SAMEJIMA K., YASUI T., 1984. Biochemical and functional characteristics of myosin from red and white muscle of chicken as influenced by nutritional stress. Agric. Biol. Chem., 48(9), 2217-2224.

CULIOLI J., BOYER C., OUALI A., 1992. Propriétés thermogélifiantes de la myosine : influence du degré de purification et du type musculaire. Sci. Aliments (In press).

DUFOUR E., OUALI A., OBLED A., DEVAL C., VALIN C., 1989. Lysosomal proteinase-sensitive regions in fast and slow skeletal muscle myosin. Biochimie, 71, 625-632.

EGELANSDAL B., FRETHEIM, K. SAMEJIMA K., 1986. Dynamic rheological measurements on heat-induced myosin gels : effects of ionic strengh, protein concentration and the addition of adenosine triphosphate and pyrophosphate. J. Food Agric., 37, 915-926.

EGELANSDAL B., MITCHELL J. R., 1987. The effect of myofibrils on the rheology of protein gels. 33rd Int. Congr. Meat. Sci. Tech. Proc., 2, 261-264.

FRETHEIM K., EGELANDSDAL B., HARBITZ B., SAMEJIMA K., 1985. Recent advances in the study of myosin and myofibril gel formation. 31st Eur. Congr. Meat Res. Work., 219-223.

FRETHEIM K., SAMEJIMA K, EGELANDSDAL B., 1986. Myosins from red and white bovine muscles : Part 1 - Gel strength (elasticity) and water-holding capacity of heat-induced gels. Food Chem, 22, 107-121.

GORNALL A., BARDAWILL C. J., DAVID M. M., 1949. Determination of serum proteins by means of biuret reaction. J. Biol. Chem., 177, 751-766.

HERMANSSON A.-M., HARBITZ O., LANGTON M., 1986. Formation of two types of gels from bovine myosin. Journal Sci. Food Agric., 37, 69-84.

ISHIOROSHI M., SAMEJIMA K., YASUI T., 1979. Heat-induced gelation of myosin : Factors of pH and salt concentrations. J.Food Sci., 44, 1280-1284.

ISHIOROSHI M., SAMEJIMA K., YASUI T., 1983. Heat-induced gelation of myosin filaments at a low salt concentration. Agric. Biol. Chem., 47(12), 2809-2816.

LAEMMLI J. K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature,227, 680-685.

NAKAI S., LI-CHANG E., HAYAKAWA S., 1986. Contribution of protein hydrophobicity to its functionality. Die Nahrung., 30, 327-336.

OFFER G., MOOS C., STAR R., 1973. A new protein of the thick filaments of vertebrate skeletal myofibrils. J. Mol. Biol., 74, 653-676.

OUALI A., GATELLIER P., DUFOUR E., 1988. Caractérisation des propriétés hydrophobes de la myosine de differents types musculaires. Viandes et produits carnés, 9, 198.

OUALI A., TALMANT A., 1990. Calpains and Calpastatin distribution in bovine, porcine ,and ovine skeletal muscles. Meat Sci., 28, 331-348.

SAMEJIMA K., ISHIOROSHI M., YASUI T., 1983. Scanning calorimetric studies on thermal denaturation of myosin and its subfragments. Agric. Biol. Chem., 47, 2373-2380.

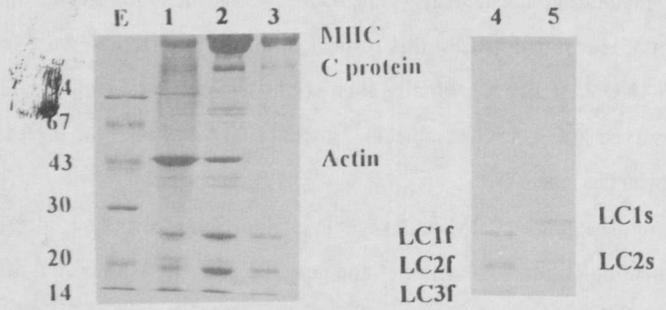
SANO T., NOGUCHI S. F., TSUCHIYA T., MATSUMOTO J.J., TSUCHIYA T.,1989. Role of F-actin in thermal gelation of fish actomyosin. J. Food Sci., 54, 800-804.

SANO T., NOGUCHI S. F., MATSUMOTO J. J., TSUCHIYA T., 1990. Effect of Ionic Strength on Dynamic Viscoelastic Behavior of Myosin During Thermal Gelation. J Food Sci., 55, 51-54,70.

SANO T., NOGUCHI S. F., MATSUMOTO J. J., TSUCHIYA T., 1990. Thermal Gelation Characteristics of Myosin Subfragments. J. Food Sci., 55, 55-58,70.

YAMAMOTO K., SAMEJIMA K., YASUI T., 1987. The structure of myosine filaments and the properties of heat-induced gel in the presence and absence of C-protein. Agric. Biol. Chem., 51, 197-203.

Figure 1 : Electrophoresis pattern of proteins (references : E. PM myofibrils : 2; PM salt-soluble fraction : 2. PM Myosin with C Protein : 3. PM pure myosin : 4. SMp pure myosin : 5.



F1 : Myofibrils
F2 : Salt-soluble fraction
F3 : myosin with C protein
F4 : Pure myosin

Figure 2 : Purification effect on PM protein gelation. 10mg/ml ; 0.6M KCl

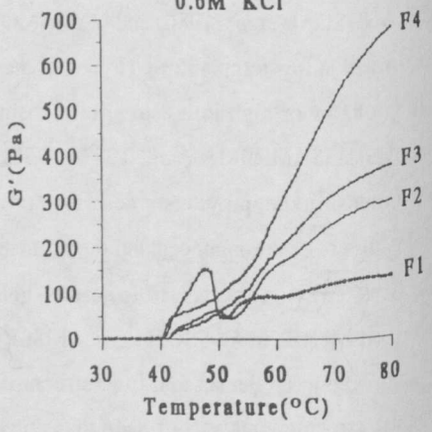


Figure 3 : Purification effect on SMp protein gelation. 10 mg/ml ; 0.6M KCl

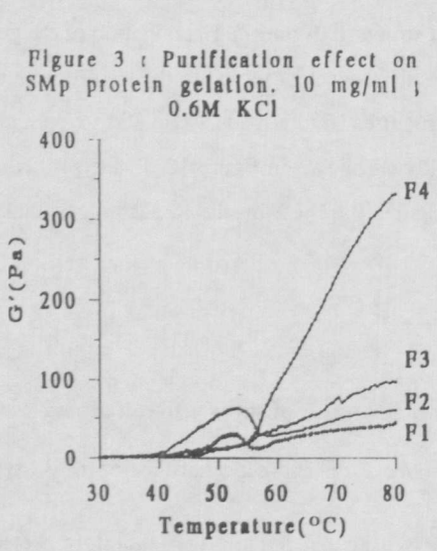


Figure 4 : Muscle type effect on myosin gelation. 10 mg/ml ; 0.6M KCl

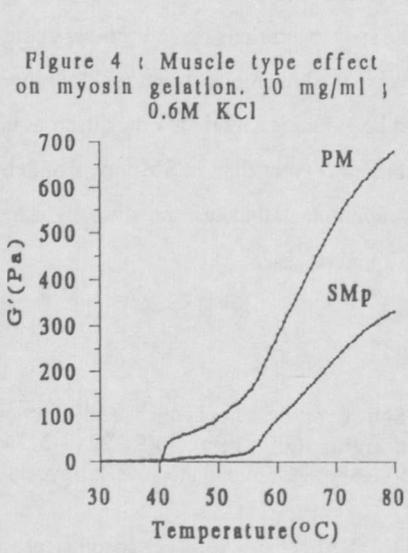


Figure 5 : Ionic strength effect on PM myosin gelation. 10 mg/ml

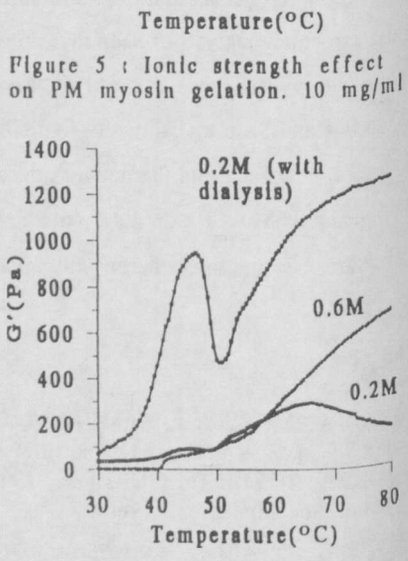


Table 1 : G' (Pa) of protein gels (10 mg/ml) at different ionic strengths and temperatures (* Peak T°are 48-49°C and 53°C for PM and SMp proteins respectively)

Muscles	Proteins	G' (Pa) at 0.6M KCl		G' (Pa) at 0.2M KCl	
		Peak T°*	80°C	Peak T°*	80°C
PM	Myofibrils	152	134	-	-
	Salt-soluble fraction	100 (13.4)	406 (40.7)	230 (22.3)	907 (66.6)
	Myosin with C protein	45 (3.6)	460 (31.3)	475 (74)	1253 (24)
	Pure myosin	62 (4.5)	620 (25.9)	790 (80)	1277 (89)
SMp	Myofibrils	30	44	-	-
	Salt-soluble fraction	52 (7.8)	49 (8.48)	-	-
	Myosin with C protein	11 (2.8)	76 (14.8)	-	-
	Pure myosin	39 (7.6)	327 (13.8)	258	496

Figure 6 : SEM micrograph of PM myosin gel (10 mg/ml) at 70°C (0.2M KCl) Bar=10µm

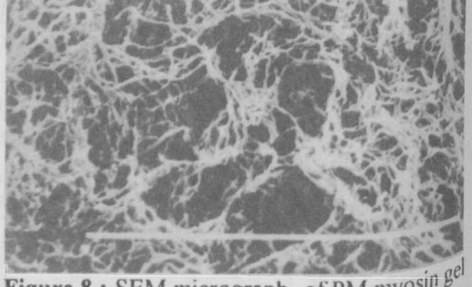


Figure 7 : SEM micrograph of SMp myosin gel (10 mg/ml) at 70°C (0.2M KCl) Bar=10µm

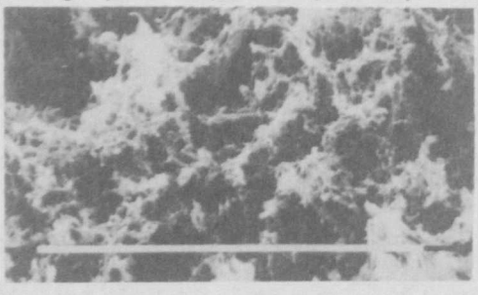


Figure 8 : SEM micrograph of PM myosin gel (10 mg/ml) at 70°C (0.6M KCl) Bar=10µm

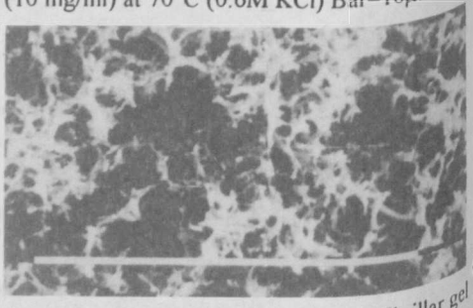


Figure 9 : SEM micrograph of SMp myosin gel (10 mg/ml) at 70°C (0.6M KCl) Bar=10µm

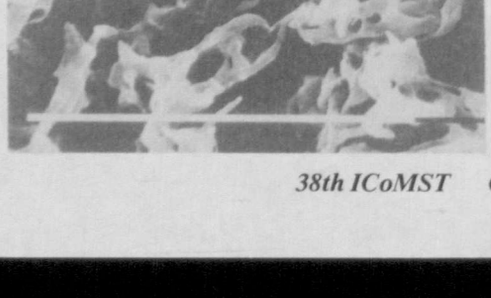


Figure 10 : SEM micrograph of myofibrillar gel (10 mg/ml) at 70°C (0.6M KCl) Bar=10µm