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Simultaneous process to isolate actomyosin and actin from post-rigor porcine skeletal muscle

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Abstract

A simultaneous actomyosin and actin isolation procedure from post-rigor porcine muscle was developed, based on differential solubility, gel filtration chromatography and extraction steps. The isolation process was evaluated by SDS-PAGE analysis and silver staining. Actomyosin and actin were isolated in a simultaneous process yielding 0.14 mg and 2.5 mg/g of meat, respectively, using a shorter purification process than others reported in the literature but with similar recoveries. Furthermore, actin preserves its polymerisation ability and both proteins, actomyosin and actin, could be used in further studies.

Keywords: Myofibrillar proteins; Myosin; Actin; Actomyosin; Purification processes; Polymerisation; Post-rigor porcine muscle

1. Introduction

The separation of the muscle constituents is necessary for various physiological and biochemical studies. In this sense, the isolation of muscle constituents allows their characterisation in order to relate them to meat quality (Bowker, Grant, Swartz, & Gerrard, 2004; Hidalgo, Padrón, Horowitz, Zhao, & Craig, 2001; Toldrá & Flores, 2004), to understand post-mortem changes (Toldrá, 2005), to the identification of contaminating proteins added to meat products (Toorop, Murch, & Ball, 1997) and to the study of the interaction phenomenon with other matrix components (Gianelli, Flores, & Toldrá, 2003). Myosin and actin, which are the major constituents of myofibrillar proteins, are not only important in muscle physiology, but they are also believed to be mainly responsible for important functional properties in food systems, such as water-holding, emulsifying capacity, binding ability and gelation, in various structured meat and meat products (Asghar, Samejima, & Yasui, 1985). Specifically, numerous methods for the isolation of these proteins have been developed, depending on the objective of the investigation, but many of them are very tedious as they include many extractions processes (Syrovy, 1984); nevertheless several rapids methods have been developed for application in the industry (Murch, Bruce, & Ball, 1992; Toorop et al., 1997).

Generally, the major myofibrillar proteins are isolated in separated processes (Syrovy, 1984). In particular, the extraction of myosin is usually initiated by the removal of the sarcoplasmic proteins through washes with diluted phosphate buffer. Subsequently, the extraction of myosin is carried out using modifications of Guba–Straub (Hermansson, Harbitz, & Langton, 1986) and Hasselback–Schneider (Dudziak & Foegeding, 1988) solutions. These buffers usually contain EDTA or EGTA to eliminate the heavy metals and to protect the enzymatic activity of the protein (Syrovy, 1984). In several modifications, DTT or β-mercaptoethanol are added at low concentrations, in order to avoid the oxidation of myosin. Furthermore, the elimination of contaminants was achieved by fractionation with ammonium sulphate and diverse chromatographic methods (Syrovy, 1984). The application of high pressure

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to enlarge the solubility of the myofibrillar proteins has been attempted (Macfarlane & Mckenzie, 1976) and some authors have even used HPLC to separate myosin from muscle extracts using high ionic strength solutions as mobile phase (Murch et al., 1992). On the other hand, the classical method to isolate actin is initiated with the elimination of the myosin and other myofibrillar proteins, using an extraction with a high ionic strength solution. Subsequently, the break of the intermolecular links in the F-actin is carried out using acetone or potassium iodide (Syrovy, 1984) and, finally, the G-actin is extracted. Subsequently, the protocol for actin purification could be divided into two steps: first, the preparation of the acetone powder and second, the extraction of the G-actin, where the ATP is added to the extraction buffer in order to maintain the actin functional integrity (Pardee & Spudich, 1982). After the extraction, the purification process is continued for several days with polymerisation and depolymerisation cycles to eliminate the contaminants (Pardee & Spudich, 1982). The following variations of this method were mainly focussed on the extraction solution, in the centrifugation speed and in the process utilised to obtain a greater purification degree, depending on the desired purity and the use required for the protein.

Moreover, most of these purification processes used pre-rigor muscle (Syrovy, 1984) but, in processed meat products, the majority of muscles used are in the post-rigor state. Furthermore, the separated constituents of post-rigor muscle would have characteristics different from those extracted from pre-rigor (Fukazawa, Nakai, & Yasui, 1970). It is important to evaluate, for example, the functional properties of proteins at the molecular level in processed products (Dudziak & Foegeding, 1988).

Summing up, the methods for isolating myofibrillar proteins are long and tedious. Therefore, the aim of this investigation was the isolation of actomyosin and actin, in an unique extraction process, from post-rigor porcine muscle, based on differential solubility, gel filtration chromatography and extraction steps, in order to obtain fractions that could be used for further studies.

2. Materials and methods

2.1. Materials

Salts, ($MgCl_2$, KCl, NaCl, NaN_3 and NaH_2PO_4) were purchased from Panreac (Barcelona, Spain), expect $CaCl_2$ (Sharlau, Barcelona, Spain) and $Na_4P_2O_7 \cdot 10H_2O$ (Riedel-de Haën, Seelze). EGTA (ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid), β-mercaptoethanol and $ATPNa_2$ (adenosine 5′-triphosphate disodium salt) were purchased from Sigma (St. Louis, MO, USA) and EDTA (ethylenediaminetetraacetic acid disodium salt 2-hydrate) from Panreac (Barcelona, Spain). Buffers Tris (Tris(hydroxymethyl)-aminoethane) and KH_2PO_4/K_2HPO_4 were purchased from Panreac (Barcelona, Spain). All the chemicals used were reagent grade (purity >98%).

Molecular weight standards of broad range for SDS-PAGE were purchased from Bio-Rad Laboratories (Richmond, CA) and include: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase-b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa) and carbonic anhydrase (31 kDa). Molecular weight standards for gel-chromatography were: myosin (450 kDa) and carbonic anhydrase (31 kDa) purchased from Sigma (St. Louis, MO, USA) and bovine serum albumin (66.2 kDa) from Roche (Mannheim, Germany).

2.2. Sample

Porcine muscle *Longissimus dorsi*, 2 d post-mortem, was acquired from local butchery. Fat and connective tissue were remove and the meat was cut in portions, packaged in vacuum bags and stored at −20 °C.

2.3. Protein purification processes

2.3.1. Simultaneous purification process

The simultaneous purification process (Fig. 1) started by washing 50 g porcine of post-rigor muscle three times with 0.1 M Tris–HCl at pH 7.0 containing 20 mM EDTA (wash buffer) in order to eliminate the sarcoplasmic proteins. Afterwards, the myofibrillar proteins were extracted with Hasselbach–Schneider solution, consisting of: 0.1 M KH_2PO_4/K_2HPO_4 at pH 6.4 with 0.6 M KCl, 10 mM $Na_4P_2O_7 \cdot 10H_2O$, 1 mM $MgCl_2$ and 20 mM EGTA (Dudziak & Foegeding, 1988) and were precipitated by diluting 1/20 with deionised water. The last pellet (M4p) was submitted to a further purification step using gel chromatography in a XK column (Ø 2.6 cm × 66.5 cm. Amersham Pharmacia Biotech, Uppsala, Sweden) filled with Sephacryl S-300 with a 10–1500 kDa fractionation range. The sample was eluted with 20 mM KH_2PO_4/K_2HPO_4 at pH 7.0 and 0.5 M NaCl as elution buffer and 80 fractions were recovered with a flow of 18 ml/h. The elution pattern was monitored by measuring the absorbance at 280 nm in a spectrophotometer Ultrospec 3000 (Pharmacia Biotech, England). The combination of the six fractions that gave the maximum absorbance constituted the fraction M8. Previously, the column was calibrated using 1 mg/ml of myosin (450 kDa), bovine serum albumin (66.2 kDa) and carbonic anhydrase (31 kDa) that were eluted in fractions 12, 30 and 39, respectively (data not shown).

In the second part of the process, the pellet obtained after the extraction with Hasselbach–Schneider solution (M3p, Fig. 1) was used to prepare the acetone powder by three successive extractions with acetone (20 vol/g pellet) for 20 min and further filtration through Whatman paper. Afterwards, the acetone powder was used for the extraction of G-actin with buffer A (20 ml/g acetone powder) containing 2 mM Tris–HCl at pH 8.0 and 0.2 mM $ATPNa_2$, 0.5 mM β-mercaptoethanol, 0.2 mM $CaCl_2$ and 0.005% NaN_3 at different extraction times. Then the fractions were

Fig. 1. Simultaneous purification process for actomyosin and actin from post-rigor porcine muscle. Wash buffer, Hasselbach–Schneider solution and buffer A are described in Section 2.

filtered through Whatman paper, yielding fractions A2sw, A3sw and A4sw.

2.3.2. Dudziak purification process

The process described by Dudziak and Foegeding (1988) was assayed to isolate myosin and actomyosin from post-rigor porcine muscle (Fig. 2). This process was carried out using the same steps as the simultaneous purification process (Fig. 1) until the M4p fraction was obtained. Later, two washing steps, using buffer D1 (0.5 M Tris–HCl at pH 7.0 with 1.5 M KCl) and buffer D2 (20 mM NaH_2PO_4 at pH 7.0 with 3.0 M KCl and 0.5 M NaCl) were performed obtaining the fractions M5p and M6s, respectively.

2.3.3. Pardee and Spudich purification process

The process described by Pardee and Spudich (1982) was assayed for the isolation of actin (Fig. 3). However, the starting material was the pellet (M3p) obtained in the simultaneous purification process (Fig. 1) instead of beginning from post-rigor porcine muscle. This process included two parts, the production of the acetone powder and the G-actin isolation. The acetone powder was obtained by extracting the M3p fraction with acetone during 20 min and repeated for five times (Fig. 3). Then, the extraction of actin from the acetone powder was performed using buffer A (described above), stirring for 30 min (and repeated twice). The supernatants obtained (A2s and A3s) were combined, yielding the fraction A4s and later it was centrifuged for obtaining the fraction A5 constituted of G-actin.

2.4. Protein concentration

The protein concentration in each fraction was determined by the bicinchoninic acid method (Smith et al., 1985) using bovine serum albumin as standard. The values are the means of four measurements.

2.5. Electrophoretic analysis

The isolated fraction purities were monitored by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), using 10% and 12% gels and stained with silver

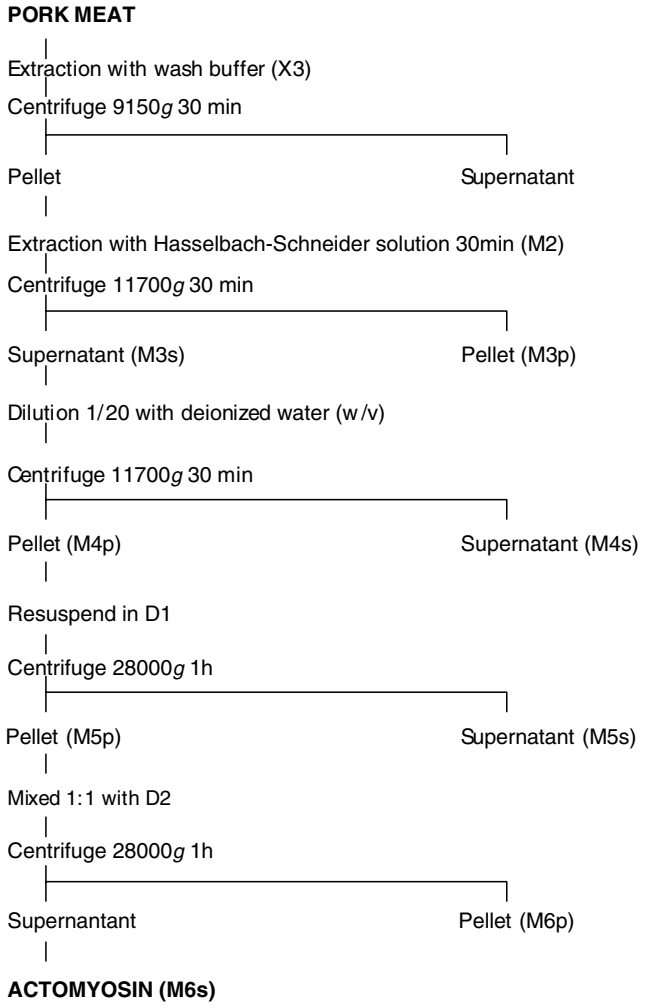

Fig. 2. Dudziak purification process for actomyosin from post-rigor porcine muscle. Wash buffer, Hasselbach–Schneider solution, D1 and D2 are described in Section 2.

(Merril, Goldman, Sedman, & Ebert, 1981). The band identification was done by comparison with molecular weight standards: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase-b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa) and carbonic anhydrase (31 kDa) (Bio-Rad Laboratories, Richmond, CA). Each lane was loaded with 5 μg and 12 μg of protein for standards and samples, respectively.

2.6. Actin polymerisation

F-actin or the polymerised form of the protein was obtained adding KCl, $MgCl_2$ and $ATPNa_2$ to the G-actin fraction to a final concentration of 50 mM, 2 mM and 1 mM, respectively. Then the mixture was kept for 12 h at 5 °C and the F-actin was separated by centrifugation at 3500 rpm for 2 min (MSE Mistral 2000, England). The polymerisation percentage was calculated by measuring the quantity of protein in the supernatant before and after centrifugation (Wang & Smith, 1994).

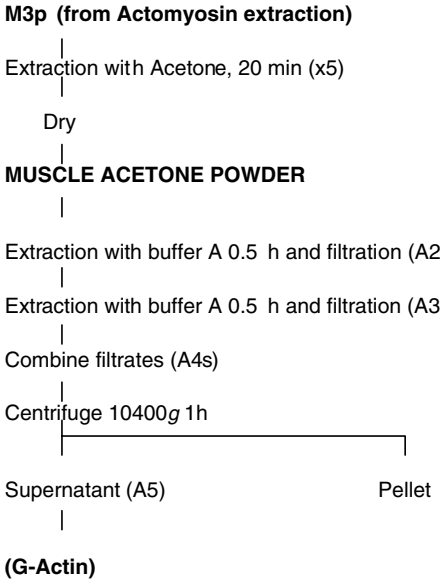

Fig. 3. Pardee and Spudich purification process for actin from post-rigor porcine muscle. Buffer A is described in Section 2.

3. Results and discussion

3.1. Actomyosin isolation

Numerous methods to isolate major myofibrillar proteins have been proposed, although these proteins have been isolated separately (Syrovy, 1984). In the present work, actomyosin has been isolated from post-rigor porcine muscle using the proposed simultaneous process and also the method proposed by Dudziak and Foegeding (1988), as shown in Figs. 1 and 2, respectively.

In the first step of the simultaneous purification process, the sarcoplasmic proteins were mainly extracted (M1a, M1b and M1c, Fig. 4A). Most of these sarcoplasmic proteins were eliminated in the first wash (M1a), as shown by the high concentration of extracted protein (Table 1). After the extraction using the Hasselbach–Schneider solution, the fractions were enriched in myofibrillar proteins (M2–M4p, Fig. 4A). This was shown by the presence of higher intensity bands at 200 kDa that correspond to the myosin heavy chain, 25 kDa that corresponded to one of the myosin light chains and 45 kDa that corresponded to actin (M2–M4p, Fig. 4A). In addition, these fractions showed other contaminant bands, such as troponins and tropomyosin at 37 and 34 kDa, respectively, and C-protein at 140 kDa (Wang & Smith, 1994). Furthermore, the SDS-PAGE gel showed a further purified fraction as the process progressed (M2–M4p, Fig. 4A) and the fractions extracted with high ionic strength (M3s and M4p, Fig. 4A) had similar protein concentrations which were around 7 mg/ml (Table 1).

On the other hand, the results from the Dudziak purification process (Fig. 2) using post-rigor porcine muscle, are shown in Fig. 4B. The SDS-PAGE gel showed that the most enriched fraction in actomyosin was M4p, as

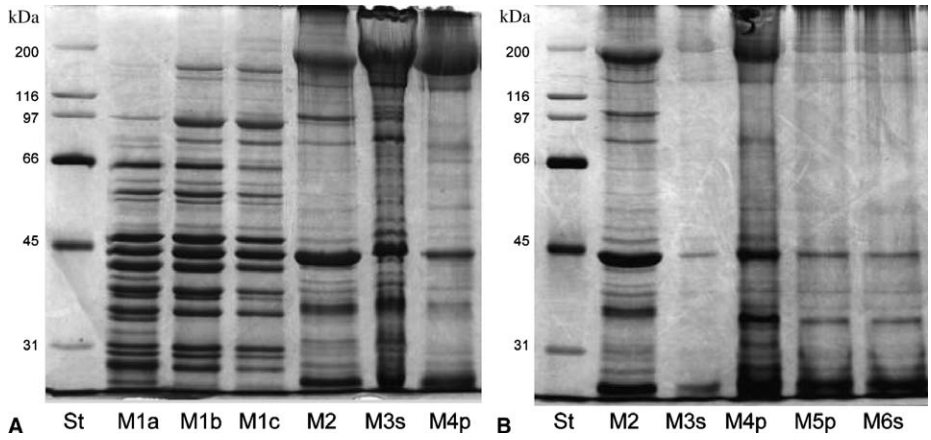

Fig. 4. Ten per cent SDS-PAGE gels of the purification processes of actomyosin stained with silver. Simultaneous purification process (A) and Dudziak purification process (B). Names of fractions in the lanes correspond to the fractions obtained by the process described in Figs. 1 and 2, respectively. St: molecular weight standards (myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase-b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa) and carbonic anhydrase (31 kDa)).

Table 1
Protein concentration obtained and total extraction times employed in the purification processes of actomyosin and G-actin isolation

Process	Protein concentration (mg/ml)							Time (h)[a]
Actomyosin	M1a	M1b	M1c	M2	M3s	M4p	M8	
Simultaneous[b]	18.4 ± 1.6	8.7 ± 0.3	2.8 ± 0.2	7.3 ± 0.2	6.5 ± 0.4	7.1 ± 0.4	0.31 ± 0.01	8
Dudziak[c]	18.3 ± 2.3					4.1 ± 0.1		10
Actin	A2s	A2sw	A3s	A3sw	A4s	A4sw		
Simultaneous	0.73 ± 0.08	0.86 ± 0.05	0.45 ± 0.00	0.42 ± 0.00	0.86 ± 0.02	0.86 ± 0.09		3
Pardee and Spudich[d]	0.75 ± 0.03				0.62 ± 0.04			4

Protein concentration is expressed as the mean value ± standard error.
[a] Approximate total extraction time employed.
[b] Simultaneous purification process, as indicated in Section 2.
[c] Dudziak purification process, as indicated in Section 2.
[d] Pardee and Spudich purification process, as indicated in Section 2.

observed by the more intense bands at 200, 25 and 45 kDa that correspond to heavy and light chains of myosin and actin, respectively. A reduction of several contaminants could be observed in the later extraction steps (M5p, Fig. 4B) but, at the same time, a reduction in the quantity of actomyosin was observed by the decrease in intensity of the bands that corresponded to myosin heavy chain and actin.

Moreover, the M4p fraction was chromatographied using Sephacryl S-300 with the aim of separating myosin from actomyosin. The chromatographic profile showed an unique peak (see Fig. 5). Different fractions of this peak contained a band that corresponded to actin at 45 kDa together with the myosin heavy chain band (200 kDa) and light chain (25 kDa) as it is shown in Fig. 6. The absence of a separated peak in fraction 12 showed that the isolation of myosin and actomyosin was not achieved and the absence of peak between fractions 30 and 39 indicated that M4p, obtained by the simultaneous process, did not contain actin (Fig. 5).

Finally, a fraction composed of highly purified actomyosin was obtained at the end of the purification process including the chromatographic step. This fraction (M8,

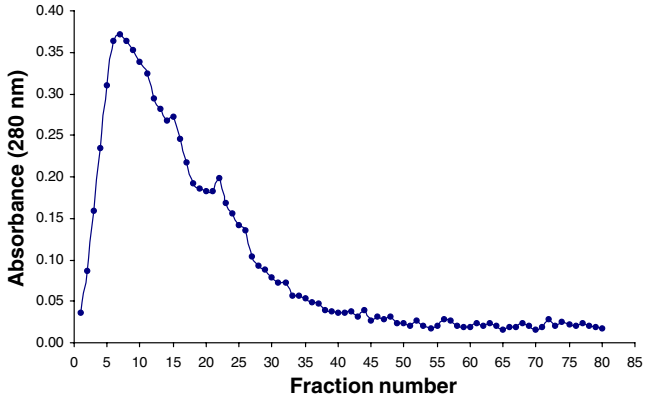

Fig. 5. Chromatographic purification of actomyosin by gel filtration using Sephacryl S-300. Previously, the column was calibrated with different proteins, including myosin (450 kDa), bovine serum albumin (66.2 kDa) and carbonic anhydrase (31 kDa) that were eluted in fractions 12, 30 and 39, respectively (data not shown).

Table 1) contained 0.306 mg/ml of protein; that means a recovery of 0.14 mg of actomyosin per gram of meat, similar to the result obtained by Dudziak and Foegeding (1988), although it was obtained in a shorter process

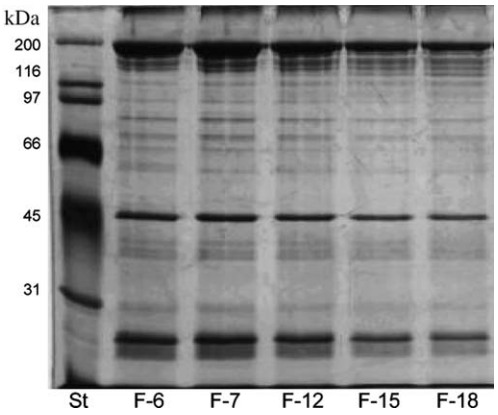

Fig. 6. Ten per cent SDS-PAGE silver stained gel of the fractions eluted from the Sephacryl S-300 column. Numbers in the lanes correspond to the fractions eluted from the column (Fig. 5). St: molecular weight standards (myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase-b (97.4 kDa), bovine serum albumin (66.2), ovalbumin (45 kDa) and carbonic anhydrase (31 kDa)).

because two washing steps with buffers D1 and D2 were eliminated (M5p and M6, Fig. 4B). On the other hand, our results could not be compared with those of other authors due to the different processes used for the extraction of these proteins, the type of muscle and animal species utilised and the use of post-rigor or pre-rigor muscle. However, in order to reduce the extraction times, Hidalgo et al. (2001) and Murch et al. (1992) developed methods for purifying myofibrillar proteins that were fast and successful for use in the industry but unsuitable for obtaining high quantity protein for further studies.

3.2. Actin isolation

Since actin was first isolated by Straub (Pardee & Spudich, 1982), several methods have been developed for its purification and most have used pre-rigor rabbit muscle (Syrovy, 1984). In this study, the simultaneous and Pardee and Spudich purification processes (Figs. 1 and 3) were applied to obtain actin from the pellet (M3p, Fig. 1) obtained in the actomyosin purification. In both processes, the acetone powder was first obtained and later the protein was extracted.

The SDS-PAGE gel of different fractions obtained using the simultaneous purification process (Fig. 7A) showed that the most intense band, corresponding to actin (45 kDa) was observed in the latest step (A4s and A4sw) and its intensity increased as the purification process progressed. On the other hand, the electrophoretic profile (Fig. 7B) for the extraction of G-actin using the Pardee and Spudich process showed that the step consisting of the combination of the supernatants A3s and A2s (Fig. 7B) only increased the contaminants in the resulting fraction (A4s) and none of these contaminants were eliminated in the later ultracentrifugation step (A5, Fig. 7B). However, the simultaneous purification process substitutes these two last steps with two longer extractions steps without combining the supernatants (A3s and A4s, Fig. 1) yielding an enriched actin fraction (Fig. 7). In addition, the filtration of fractions through Whatman paper did not reduce contaminant bands (A2sw, A3sw and A4sw, Fig. 7A).

Table 1 shows that the final fraction (A4sw), enriched with actin, had a protein concentration of 0.86 mg/ml. This means a recovery of 18.6 mg of G-actin per gram of acetone powder or 2.5 mg/g of meat (post-rigor porcine muscle) while Pardee and Spudich (1982) obtained a yield of 10 mg of actin per gram of acetone powder, although they suggested that a greater amount (even 30 mg) could be achieved.

On the other hand, Kuroda (1982) obtained G-actin from myofibrillars without acetone treatment but these proteins lost polymerisation ability after prolonged extraction. In order to check the functionality of the isolated actin, the polymerisation of A4sw fraction was carried out. The protein conserved the polymerisation ability but the obtained percentage (64%) was lower than that

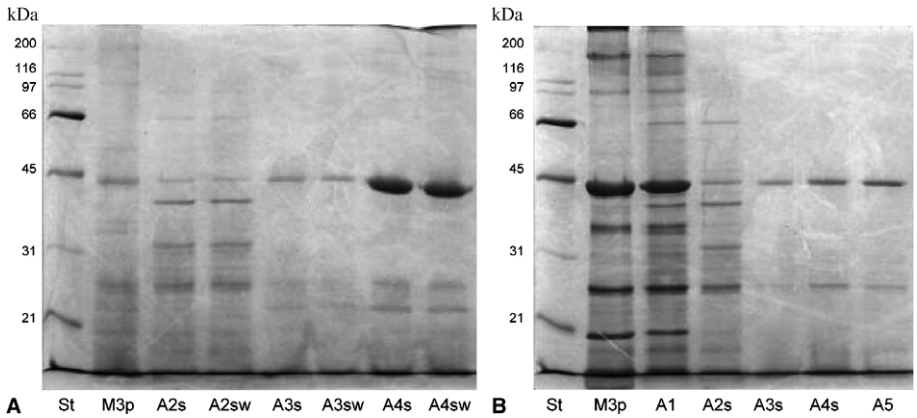

Fig. 7. Twelve per cent SDS-PAGE gels of the purification processes of actin stained with silver. Simultaneous purification process (A) and Pardee and Spudich purification process (B). Name of fractions in the lanes correspond to the fractions obtained by the process described in Figs. 1 and 3, respectively. St: molecular weight standards (myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase-b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa) and carbonic anhydrase (31 kDa)).

achieved by Wang and Smith (1994), probably due to the use of a less purified fraction and different animal species.

Summing up, actomyosin was purified using a modified protocol from Dudziak and Foegeding (1988) where post-rigor porcine muscle was used instead of pre-rigor muscle but obtaining a similar recovery. In the same purification process, G-actin was isolated from the same original sample and following a modification of the Pardee and Spudich (1982) process. However, the recovery was higher than that obtained by these authors, probably due to a less purified fraction, although its polymerisation ability was conserved, which made it appropriate for further studies. Nevertheless, in comparison to the initial purification protocols, the developed simultaneous purification process was shorter which is an advantage for obtaining purified myofibrillar proteins for later studies.

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