LATION AND CHARACTERIZATION OF PROTEASOMES (PROSOMES) FROM RABBIT MUSCLES

ie-Noëlle POUCH, Gérard BOISSONNET, Yves BRIAND and Hans-Peter SCHMID
versité Blaise Pascal, Clermont-Ferrand, 24 Avenue des Landais, F-63177 Aubière Cédex, France

MARY

Proteasomes (prosomes) are involved in various intracellular metabolisms of degradation. They have protease, peptidase, as well Nase activity and block the in vitro translation of various viral RNAs.

A combination of ultracentrifugation low- and high pressure chromatography was used to purify proteasomes from rabbit scles. They eluted from Mono Q columns (FPLC-Pharmacia) in Tris-buffered solutions at 320 mM KCl while proteasomes from calf- e eluted at 480 mM KCl. Thus the ionic charge of both particles is quite different. Proteasomes of rabbit muscles consist of a specific f proteins which band in Laemmli polyacrylamide gels in the range of 22 000 - 33 000 Da. In two dimensional gel systems its protein ponents migrated rather different than proteins of calf liver or rabbit liver proteasomes.

RODUCTION

Proteasomes (Prosomes) are large particles with sedimentation coefficients of about 19S (Schmid et al., 1984 ; Martins de Sa et 1986 ; Arrigo et al., 1988). They have a typical cylinder shaped structure and consist of a specific set of proteins which band in SDS- mmli gels in the range of 19 000 - 35 000 dalton. Some of them e.g. the 27 000 dalton protein were highly conserved during ution where others vary from species to species (Grossi de Sa et al., 1988 ; Kreutzer-Schmid et al., 1990). Proteasomal proteins are osylated (Tomek et al., 1988). A matter of controversity is the content of RNA at least part of these particles contain small RNA hmid et al., 1984 ; Dineva et al., 1989). Proteasomes (Prosomes) are widely distributed in eucaryotes ranging from yeast to human lmann et al., 1989 ; Schliephacke et al., 1991), suggesting a fundamental role in cellular events.

Proteasomes appear to function as critical parts of an ATP dependent proteolytic pathway, they catalyse the break down of iquinated proteins (Driscoll et al., 1990 ; Kanayama et al., 1991) and they have RNase activity (Tsukahana et al., 1989). Proteasome pression has been found abnormaly high in human leucemia cells (Kumatori et al., 1990). Others reported that deletion of certain teasomal proteins was lethal in yeast (Fujiwara et al., 1990 ; Heinemeyer et al., 1991). Finally we have shown that proteasomes hibit the in vitro translation of viral mRNA but not of cellular mRNA (Horsch et al., 1989). Here we present a fast and effective method solating proteasomes of rabbit muscle tissue using a combination of ultracentrifugation low and high pressure chromatography.

TERIAL AND METHODS

l fractionation procedure

Leg muscles were removed from a freshly slaughtered rabbit, cut into small pieces and washed several times with cold buffer taining 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 0.1 mM EDTA (pH 7.4), 50 mM KCl (washing buffer). connective tissue was removed from the muscle fragments. Portions of 20 grammes were homogenized in a polytron (Kinematica, tris, Switzerland) with 3 volumes of buffer containing 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 0.1 mM TA (pH 7.4), 50 mM KCl, 200 mM sucrose.

Homogenates were filtered through Miracloth and portions of 20 ml were then further homogenized by 20 strokes in a Dounce mogenizer and centrifuged for 15 min at 1500 rpm at 4°C (GSA rotor, Sorvall RC 2-B) and the remaining supernatant was centrifuged 15 min at 10 000 rpm at 4°C (GSA rotor, Sorvall RC2-B). The supernatant from this sedimentation was centrifuged again for 20 min 16000 rpm (SS 34 rotor, Sorvall RC-2B) to eliminate mitochondria and cellular membranes. The post-mitochondrial supernant was nsfered to Beckman Quickseal tubes (Ti 45 rotor) containing a cushion of 30% sucrose in wash buffer and centrifuged 2 h at 42 000 m at 4°C to sediment polysomes and other particles larger than 80S. The postribosomal supernatant (PRS) was centrifuged again in ckman Quick seal tubes (Ti 45 rotor) containing a cushion of 10 ml of 30% sucrose in wash buffer (19 h, 42 000 rpm, 4°C). The ments containing post ribosomal particles (PRPs) were frozen at -80°C or resuspended in TBK 240 (20 mM Tris-HCl (pH 7.4) 5 mM $MgCl_2$, 7 mM 2 mercaptoethanol, 240 mM KCl) and immediately subjected to different chromatographic procedures.

e and two dimensional protein gel electrophoresis

One dimensional SDS-PAGE of proteins was performed according to Laemmli (1971) and two dimensional protein gel ctrophoresis as described by O'Farrel et al. (1977). Molecular weight markers were phosphorylase b (90 000 dalton), bovine serum umin (67 000 dalton), ovalbumine 43 000 dalton, carboanhydrase (29 000 dalton), soybean trypsin inhibitor (20 000 dalton) and albumin (14 000 dalton).

SULTS AND DISCUSSION

To investigate the structure and function of proteasomes in muscle cells we developed a fast and effective method for the ification of these particles.

We started the purification with about 160 g muscle tissu. The 210 000 g sediments containing ribosomal subunits, free mRNPs, small cytoplasmic RNPs and proteins larger than 10S (Postribosomal particles : PRPs) were purified by anion exchange chromatography. Approximately 96-120 A_{280} units of PRP suspension (the whole preparation) was applied to 200 ml of Q-Sepharose fast flow anion exchanger (Pharmacia) in a C26/40 column (Pharmacia-LKB) equilibrated in TBK 240.

Particles and unbound proteins were washed off with the same buffer until the absorbance of 280nm reached a constant value near the baseline. Fractions containing proteasomes were eluted by a step gradient to 600 mM (Fig. 1, peak 2). Fractions 50-60 were pooled (Fig. 1) diluted with FPLC buffer A (20 mM Tris-HCL (pH 7.4), 5 mM $MgCl_2$, 7 mM 2-mercaptoethanol) to a salt concentration of 300 mM KCl and applied to a fast protein liquid chromatography (FPLC) Mono Q column (HR 5.5, Pharmacia LKB) equilibrated in FPLC buffer A without KCl. Then a linear salt gradient up to 600 mM KCl was formed. Proteasomes were eluted at 320 mM with a pronounced peak of abscorbance in fraction 22-24 (Fig. 2, peak 1 ; Fig. 3, lane 2) ; other particles eluted at higher salt concentrations. Interestingly peak n° 2 consist of almost one protein which migrates in the range of 90 000 dalton.

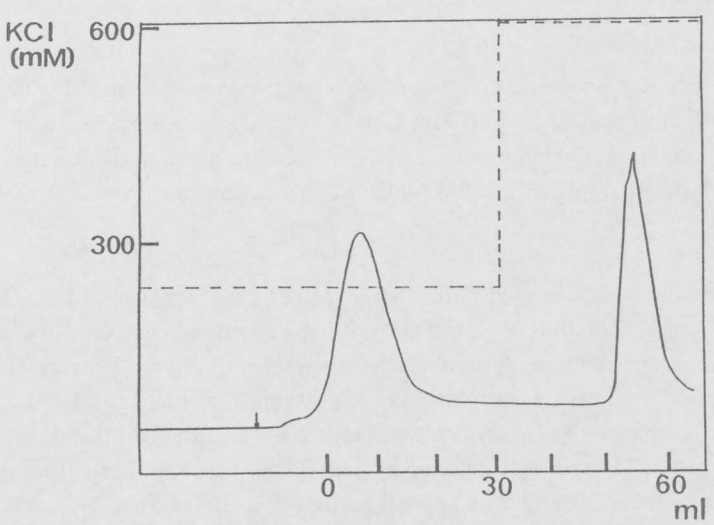
To concentrate the proteasomes fractions 22-24 from 3 Mono Q runs were collected, diluted to a final concentration of 160 mM KCl and again loaded on a Mono Q column.

After a short wash with TBK 160, bound particles were eluted by a step gradient to 480 mM KCl. Under these conditions, all particles bound on the exchanger were recovered in 1 ml, the void volume of the column. Finally proteasomes were purified to homogeneity by gel filtration using a FPLC Superose 6 column (HR 10/30, Pharmacia-LKB) equilibrated with TBK 480. Proteasomes were eluted as a homogenous fraction with a retention of 13 ml, which corresponds well with a molecular weight of 660 000 dalton and a sedimentation constant of 19S estimated by ultracentrifugation.

Proteasomes from rabbit muscles purified according to the FPLC procedure revealed a characteristic protein pattern when subjected to two dimensional protein gel electrophoresis. With this method at least 14 different protein spots in the range of 22 000-33000 Da could be identified demonstrating the complexity of these particles (Fig. 4). In comparison to proteasomes isolated from liver (calf, mouse, rabbit) the electrophoretic mobilities of proteasomal proteins of rabbit muscle are quite different.

Most characteristic is the wide gap between two acid proteins (left) and the other protein components (right).

This might reflect the lower affinity of muscle proteasomes to the Mono Q anionic exchanger ; they eluted at 320 mM KCl, while proteasomes of liver cells eluted much higher at 480 mM KCl (Tomek et al., 1990). If this is due to a different degree of glycosylation or other post translational modifications is under investigation.



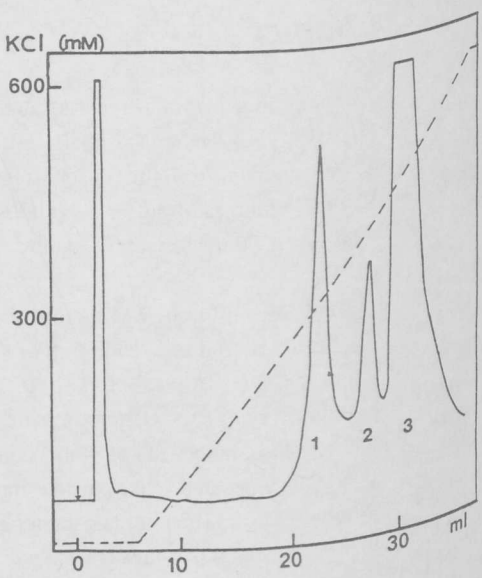
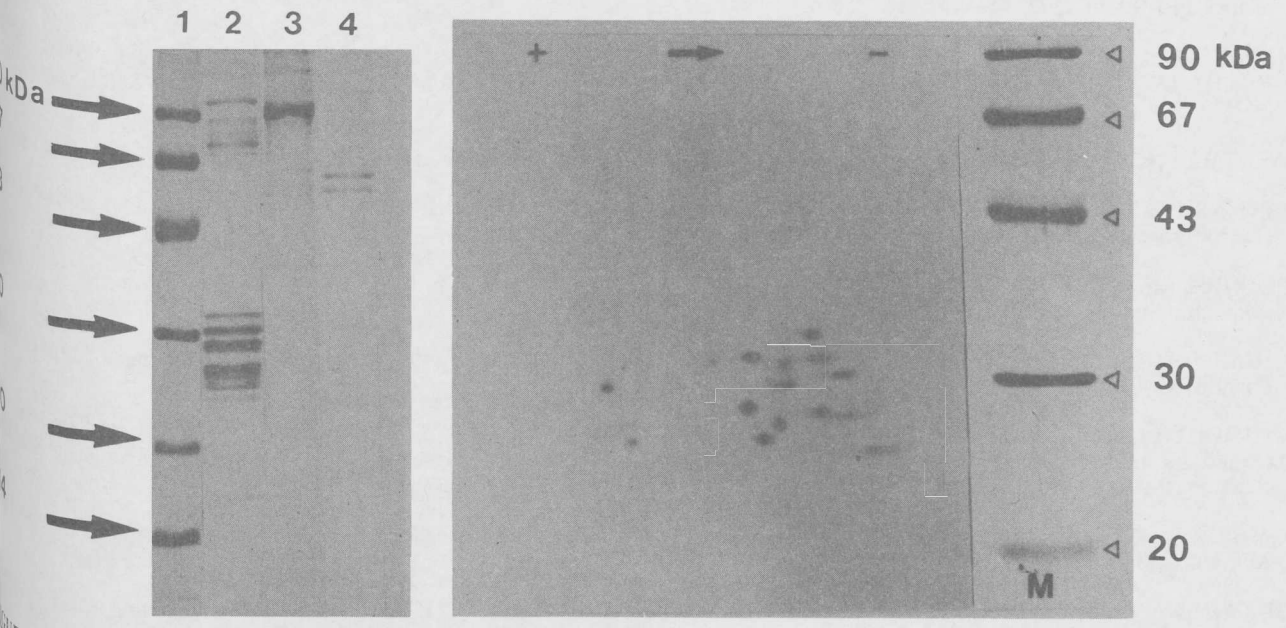


FIGURE 1 (left) Chromatogram of particles eluted from Q Sepharose fast-flow.

PRP suspension was applied to a Q-Sepharose fast-flow column equilibrated in TBK 240 : unbound particles eluted at 240 mM KCl while bound particles were eluted by a step gradient to 600 mM KCl in Tris-HCl buffer (pH 7.4). Dashed line, KCl concentration ; solid line, absorbance at 280 mM (A_{280}).

FIGURE 2 (right) Chromatogram of particles eluted from the FPLC Mono Q HR 5/5 column.

Particles eluted at 600 mM KCl from the Q Sepharose fast-flow were diluted to a final concentration of 200 mM KCl and applied to FPLC Mono Q HR 5/5 column. Bound particles were eluted with a linear salt gradient from O to 600 mM KCl in Tris-HCl buffer (pH 7.4). Dashed line, KCl concentration ; solid line, absorbance at 280 nm (A_{280}).



URE 3 (left) Protein composition of the peak fractions eluted from the FPLC Mono Q HR 5/5 column.

Fractions of peak No 1,2,3 (fig. 2) were analyzed by one dimensional SDS-PAGE on 12.5% polyacrylamide gels and stained Coomassie brilliant blue, molecular weight markers are in kilodaltons.

URE 4 (right) Two dimensional protein gel electrophoresis of purified proteasomes.

Proteasomes purified by gel filtration (data not shown) were analysed by two dimensional PAGE. Proteins were stained with omassie brilliant blue, molecular weight markers are in kilodaltons.

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