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# Purification and Characterization of a 15S ATPase from Chick Skeletal Muscle

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**An ATPase complex has been purified to apparent homogeniety from the extract of chick skeletal muscle using conventional column chromatographies and glycerol density gradient centrifugation. This enzyme has a sedimentation coefficient of 15S as determined by the gradient centrifugation and therefore is referred to as the 15S ATPase. It behaves as a 600-kDa molecule upon gel filtration analysis using a Superose-6 column. However, the ATPase runs as a 95-kDa polypeptide when analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Thus, the ATPase is likely to consist of six identical subunits of 95 kDa. It has a $K_m$ value of 0.6 mM for ATP and is maximally active at pH 9.**

**KEY WORDS: ATPase, hexameric complex, chick skeletal muscle**

An ATP-dependent protease responsible for the breakdown of ubiquitinated proteins has been first purified from human reticulocytes and then from a variety of other animal sources (Hough *et al.*, 1986, 1987; Waxman *et al.*, 1987; Hoffman *et al.*, 1992; Lee *et al.*, 1993; Ugai *et al.*, 1993). This enzyme complex has a sedimentation coefficient of 26S and consists of a proteolytic core, called 20S proteasome containing multiple subunits of 20-32 kDa, and a regulatory component containing a set of larger polypeptides of 42-110 kDa. The 26S protease complex, however, could not be found when reticulocytes were depleted of ATP (Ganoth *et al.*, 1988). In addition, it has been suggested that ATP hydrolysis is required for continuous action of the 26S protease complex in the degradation of the ubiquitin conjugated proteins (Armon *et al.*, 1990). Thus, ATP appears to play an essential role in both the assembly of the 26S protease complex and its proteolytic function. However, little is known about the protein component(s) of the 26S protease complex that catalyzes ATP hydrolysis, although the 26S protease complex as a whole is known to exhibit considerable ATPase activity (Armon *et al.*, 1990).

During purification of the 26S protease complex from chick skeletal muscle (Lee *et al.*, 1993), we noticed an ATPase activity which is initially coeluted with the 26S protease complex upon gel filtration but can be dissociated from it by later purification steps. Therefore, the present studies were undertaken to purify the ATPase and to chracterize its physical and biochemical properties.

## Materials and Methods

### Materials

[$\gamma$-$^{32}$P]ATP (3 Ci/mmol) was purchased from Amersham, N-succinyl-Leu-Leu-Val-Tyr-7-amido-

4-methylcoumarin (Suc-LLVY-AMC) from Peptide Institute (Osaka, Japan), BioGel A-1.5m from BioRad, and Q-Sepharose from LKB-Pharmacia. All other chemicals were obtained from Sigma unless otherwise indicated.

### Assays

ATP hydrolysis was assayed as described by Armon *et al.* (1990). Briefly, reaction mixtures (50 $\mu$l) containing 25 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 50 $\mu$g heat-denatured bovine serum albumin (BSA), 1 $\mu$M [$\gamma$-$^{32}$P]ATP and 1 mM unlabeled ATP were incubated with enzyme samples for 1-4 hr at 37°C. The reaction was then terminated by successive additions of 100 $\mu$l of 0.1 N HCl, 100 $\mu$l of a solution of 2 mg/ml BSA, and 250 $\mu$l of a 10% (w/v) suspension of activated charcoal. The samples were stirred on a vortex mixer, incubated on ice for 15 min, and centrifuged at 15,000 $\times$g for 3 min. Radioactivity in the supernatants was determined by measuring Cerenkov radiation in a liquid scintillation counter. Protein concentration was measured using BSA as a standard as described by Bradford (1976).

The cleavage of fluorogenic peptides was determined by incubating reaction mixtures (0.1 ml) containing appropriate amounts of enzyme samples, 25 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.5 mM DTT, 1 mM ATP, and 0.1 mM Suc-LLVY-AMC at 37°C for 30-60 min. The reaction was then stopped by adding 100 $\mu$l of 10% SDS and 800 $\mu$l of 0.1 M Na-borate (pH 9.1), and release of fluorophores was measured as described (Ahn *et al.*, 1991).

### Electrophoretic analysis

Gel electrophoresis in the presence of SDS was performed in 8% (w/v) polyacrylamide slab gels (Laemmli, 1970). Electrophoretic analysis under a nondenaturing condition was carried out in 4.5% slab gels as described by Hough *et al.* (1987), except that the buffer used for gel polymerization was 360 mM Tris-HCl (pH 8.3) containing 320 mM boric acid. In addition, 0.5 mM ATP was included in the sampling buffer containing 70 mM Tris-HCl (pH 7), 3 mM $MgCl_2$ and 5% glycerol. Proteins in the gels were visualized by silver staining (Harlow and Lane, 1988).

## Results and Discussion

### Purification of the 15S ATPase

Chick pectoralis muscle (100 g) was minced and homogenized in a Waring blender in 400 ml of buffer-A [25 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP] containing 0.25 M sucrose. The homogenates were centrifuged at 100,000 $\times$g for 1 hr, and the resulting supernatants were again centrifuged at 100,000 $\times$g for 12 hr. The precipitates were suspended in 6 ml of buffer-A containing 20% glycerol. The suspensions were loaded on a BioGel A-1.5m column (3 $\times$ 130 cm) equilibrated with the same buffer. Fig. 1A shows that a peak of ATPase activity is eluted in the fractions corresponding to a molecular mass of 1,300-1,500 kDa. Furthermore, the ATPase peak was closely overlapped with the activity peak of 26S protease complex against Suc-LLVY-AMC. Therefore, we initially thought that the 26S protease complex might be responsible for the ATPase activity. The peptide-degrading activity that appeared at the right-side shoulder of the 26S protease peak was dramatically increased when assayed in the presence of SDS, indicating that the activity is due to the 20S proteasome (data not shown).

To determine whether the ATPase activity is indeed due to the 26S protease complex or can be separable from each other, the fractions under the bar in Fig. 1A were pooled and applied to a Q-Sepharose column (1.5 $\times$ 5.7 cm) equilibrated with buffer-A containing 20% glycerol. The column was washed extensively with the same buffer containing 0.25 M NaCl, and the proteins bound to the column were eluted with a linear gradient of 0.25-0.5 M NaCl (total 100 ml). As shown in Fig. 1B, the ATPase activity was separated into two peaks: one remained overlapped with the peptide-degrading activity and the other was eluted alone at about 0.4 M NaCl. When the latter ATPase peak was again chromatographed on the BioGel A-1.5m column, it was recovered in the fractions corresponding to about 600 kDa but not to 1,300-1,500 kDa (data

not shown). These results suggest that the ATPase peak with 600-kDa size is distinct from the 26S protease complex and may be dissociated into the 600-kDa molecules under the purification conditions, such as in the presence of salt. However, it is also possible that there may exist certain specific interaction between the 26S protease complex and the ATPase.

The fractions under the bar in Fig. 1B were pooled, concentrated by ultrafiltration on a PM-10 membrane (Amicon), and dialysed against buffer-A containing 7% glycerol. The samples were then subjected to sedimentation on a linear gradient of 10-40% glycerol in buffer-A. After centrifugation at 23,000 rpm for 36 hr in a SW-28 rotor (Beckman), fractions of 1.5 ml were collected. As shown in Fig 2A, the ATPase activity as well as the peptide-degrading activity were separated into two peaks. In order to identify each of the activities, aliquots of the gradient fractions were subjected to gel electrophoresis under a nondenaturing condition (Fig. 2B). These results clearly show that the 26S protease complex is responsible for the first peaks of the ATPase and peptide-degrading activity and the second peptidase peak is due to the 20S proteasome. These results also demonstrate that the newly found, slow-migrating band with a sedimentation coefficient of about 15S is responsible for the

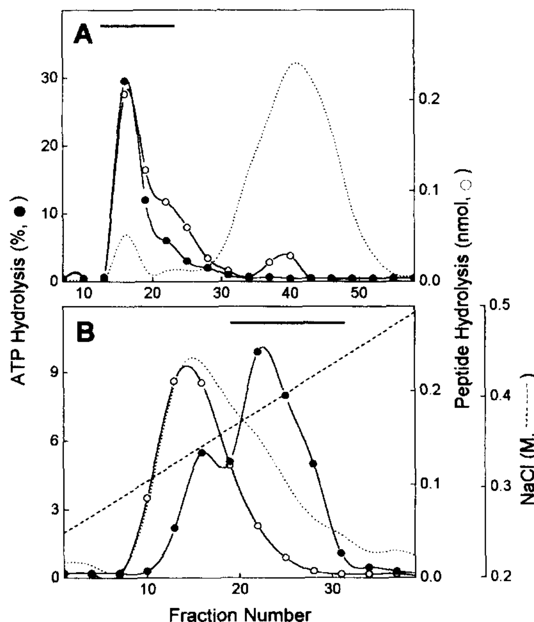


**Fig. 1.** Separation of an ATPase activity from the 26S protease complex using (A) BioGel A-1.5m and (B) Q-Sepharose column chromatographies. (A) Proteins (415 mg) from the ultracentrifugation step were chromatographed on a BioGel A-1.5m column as described in the text. Fractions of 10 ml were collected at a flow rate of 40 ml/hr and assayed for their ability to hydrolyze [$\gamma$-$^{32}$P]ATP (●) and Suc-LLVY-AMC (○) as described under Materials and Methods. (B) Fractions under the bar in A were pooled and loaded on a Q-Sepharose column. Fractions of 1.5 ml were collected at a flow rate of 30 ml/hr and assayed for their ATPase (●) and peptidase activities (○) as above. The dotted and slashed lines indicate protein profile and NaCl gradient, respectively.

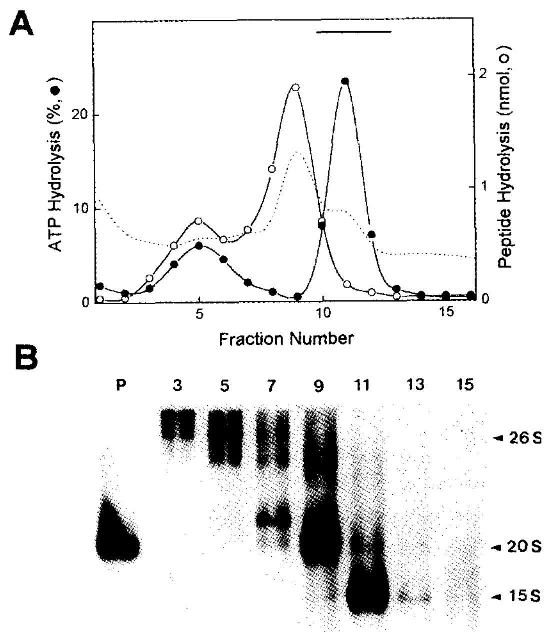


**Fig. 2.** Separation of the ATPase activity by glycerol density gradient centrifugation. (A) Fractions under the bar in Fig. 1B were pooled, concentrated, loaded onto a 10-40% glycerol density gradient (32 ml/tube) and centrifuged as described in the text. Fractions of 1.5 ml were collected and assayed for their ability to hydrolyze ATP (●) and Suc-LLVY-AMC (○). (B) Aliquots of the fractions were also subjected to polyacrylamide gel electrophoresis under a nondenaturing condition as described under Materials and Methods. Proteins in the gel were then visualized by silver staining. The letter P indicates the purified 20S proteasome, and the numerals on top of the gel show the fraction numbers.

second peak of the ATPase activity. Therefore, the newly found ATPase activity is henceforth referred to as the 15S ATPase.

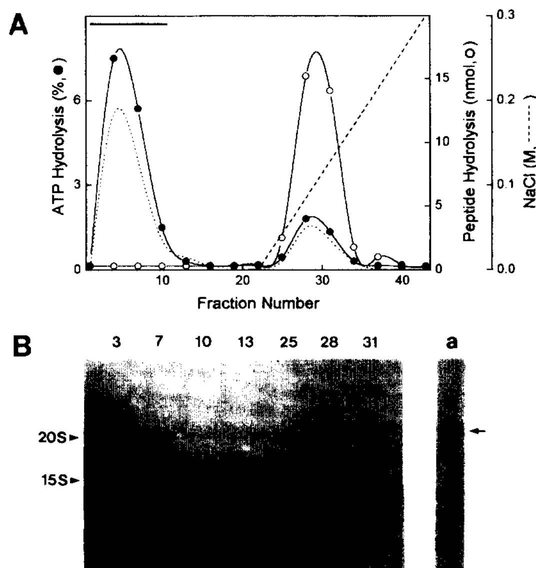


**Fig. 3.** Elution of the 15S ATPase from a heparin-agarose column. (A) Fractions under the bar in Fig. 2A were pooled and loaded on a heparin-agarose column as described in the text. Fractions of 1 ml were collected and assayed for their ability to hydrolyze ATP (●) and Suc-LLVY-AMC (○). (B) Aliquots of the fractions were also subjected to polyacrylamide gel electrophoresis under a nondenaturing condition (left pannel). An aliquot of the pooled fractions under the bar in A were also subjected to electrophoresis on a 8% gel but in the presence of SDS (right pannel). Proteins in the gels were visualized by silver staining.

In order to purify further the 15S ATPase, the fractions under the bar in Fig. 2A were pooled and loaded on a heparin-agarose column (1 × 25 cm) equilibrated with buffer-A containing 20% glycerol. After collecting 1 ml fractions of unbound proteins, the column was eluted with a linear gradient of 0-0.3 M NaCl. Fig. 3A shows that nearly all of the peptide-degrading activity of 20S proteasome was recovered in the gradient fractions. On the other hand, approximately 80% of the total ATPase activity was eluted in the flow-through fractions and the remaining activity was in the NaCl eluents. These results suggest that the 15S ATPase may consist of two population, which may differ from each other such as by certain covalent modifications.

We then determined the purity of the 15S ATPase by electrophoresis of the peak fractions under a nondenaturing condition. Only the 15S ATPase band could be seen in the flow-through fractions (Fig. 3B, left pannel), indicating the 15S ATPase was purified to apparent homogeneity. In order to confirm its purity further, the fractions under the bar in Fig. 3A were pooled and again subjected to electrophoresis but in the presence of SDS. The right pannel in Fig. 3B shows that the 15S ATPase is mainly comprised of 95-kDa subunits. The minor band of about 83 kDa appears to be a limited cleavage product of the major 95-kDa polypeptide (see the accompanying paper, Shim *et al.*, 1994). The purified 15S ATPase was concentrated by ultrafiltration and kept frozen at −70°C for further use. Summary of

**Table 1.** Summary of purification procedures.

| Steps | Protein (mg) | Total activity (units[a]) | Specific activity (units/mg) |
|---|---|---|---|
| Muscle extract | 4,813 | ND[b] | ND |
| Ultracentrifugation | 415 | ND | ND |
| BioGel A-1.5m | 10 | ND | ND |
| Q-Sepharose | 0.8 | 475 | 594 |
| Glycerol density gradient centrifugation | 0.1 | 175 | 1,750 |
| Heparin-agarose | 0.04 | 113 | 2,825 |

[a]One unit of the activity is defined as nmol Pi released from [$\gamma$-$^{32}$P]ATP per 1 hr.

[b]ND, not determined because of the presence of other ATP-cleaving enzymes in the protein samples, such as the 26S protease complex.

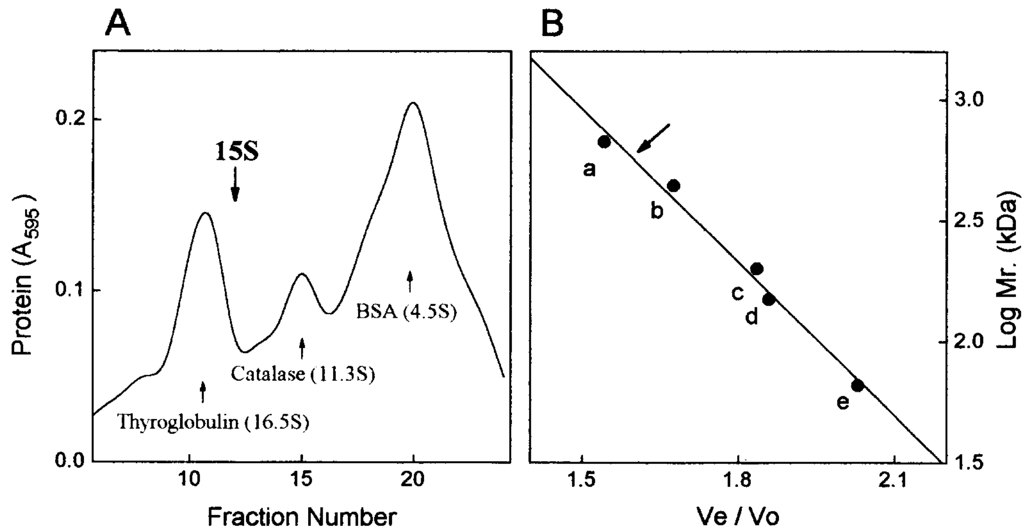


**Fig. 4.** Determination of the sedimentation coefficient and native size of the purified ATPase. (A) Fractions under the bar in Fig. 3A were pooled and concentrated by ultrafiltration, and an aliquot (50 μg) of the sample was subjected to ultracentrifugation on a 10-40% glycerol gradient as in Fig. 2 but in the presence of marker proteins with known sedimentation coefficients. (B) An aliquot (50 μg) of the same sample was also chromatographed on a Superose-6 column (1 × 30 cm) equilibrated with buffer A containing 10% glycerol. Fractions of 0.5 ml were collected at a flow rate of 15 ml/hr. The size markers used were: a, thyroglobulin (669 kDa); b, apoferritin (443 kDa); c, β-amylase (200 kDa); d, alcohol dehydrogenase (150 kDa); e, BSA (66 kDa).

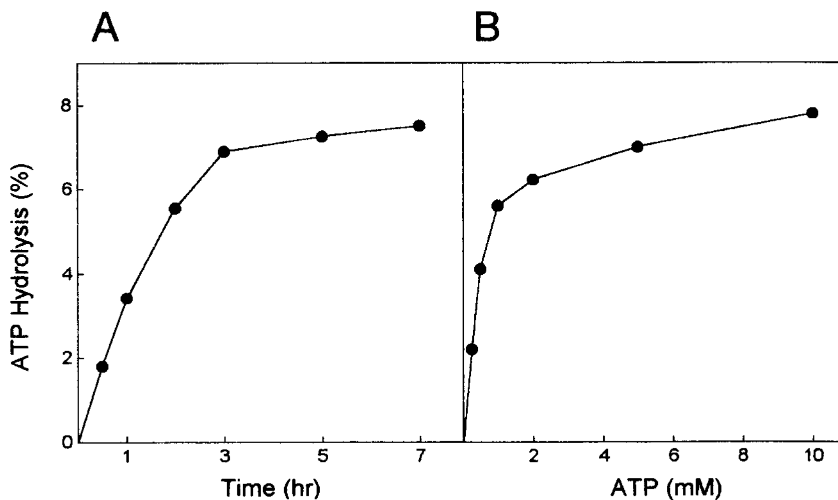


**Fig. 5.** Incubation time- and ATP dose-dependency of the activity of the purified 15S ATPase. (A) The purified enzyme (1 μg) was incubated at 37°C for the indicated periods. The release of radioactive free phosphates from [$\gamma$-$^{32}$P]ATP was then determined as described under Materials and Methods. (B) Incubations were also performed as above but for 2 hr in the presence of the increasing amounts of ATP.

the purification is shown in Table 1.

**Physical and biochemical properties of the 15S ATPase**

In order to determine more precisely the structural feature of the ATPase, the purified protein was again subjected to ultracentrifugation on a 10-40% glycerol density gradient together with the marker proteins with known sedimentation coefficients. Fig. 4A shows that the ATPase runs as a 15S particle, indicating that it is an oligomeric complex. We also performed the gel filtration analysis of the purified ATPase using a Superose-6 column. As shown in Fig. 4B, it again ran as a 600-kDa protein molecule. Thus, the 15S ATPase appears to be a homo-hexameric complex of 95-kDa subunits.

**Table 2.** Nucleotide specificity of the 15S-ATPase.

| Nucleotides | % Relative Activity |
|---|---|
| [$\gamma$-$^{32}$P]ATP alone | 100 |
| With ATP | 13 |
| With CTP | 31 |
| With GTP | 60 |
| With UTP | 82 |

ATP hydrolysis was assayed by incubating 1 $\mu$g of the purified 15S ATPase with 1 $\mu$M [$\gamma$-$^{32}$P]ATP and various unlabeled nucleotides (0.5 mM) at 37°C for 2 hr. The activity seen without any unlabeled nucleotide was expressed as 100%.

To examine the time-dependent hydrolysis of ATP by the 15S ATPase, the purified enzyme was incubated with [$\gamma$-$^{32}$P]ATP for various periods. As shown in Fig. 5A, ATP-hydrolysis increased linearly with time for at least 2-3 hr. We also examined the effect of increasing concentrations of ATP (Fig. 5B). Using a double reciprocal plot of these data, the $K_m$ for ATP was estimated to be about 0.6 mM. For determination of the nucleotide specificities of the enzyme complexes, hydrolysis of [$\gamma$-$^{32}$P]ATP was assayed in the presence of excess amounts of various non-radioactive nucleoside triphosphates. Table 2 shows that competition was the greatest by ATP followed in order by CTP, GTP and UTP, suggesting that the 15S ATPase preferentially uses ATP as the substrate but without strict specificity. This enzyme is maximally active at pH 9, but is almost completely inactivated at pH below 5 and above 11 (Fig. 6).

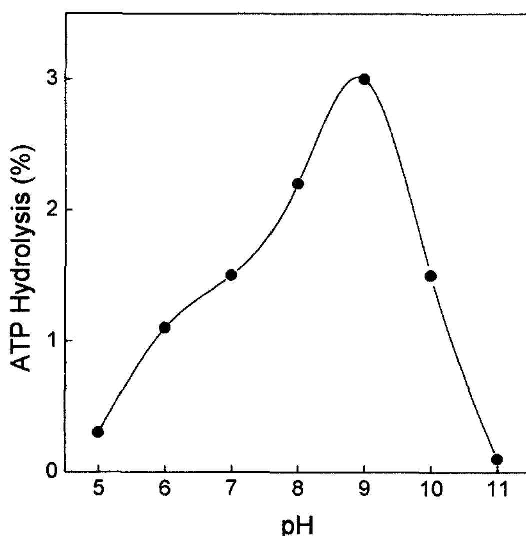


**Fig. 6.** pH-dependence of the activity of the 15S-ATPase complex. The activity of the 15S ATPase was determined at various pHs by incubation of 1 $\mu$g of the purified enzyme with [$\gamma$-$^{32}$P]ATP for 2 hr at 37°C. The buffers used were sodium acetate for pH 5, 2-(N-morpholino)ethane sulfonic acid (MES) for 6, morpholinopropane sulfonic acid (MOPS) for 7, Tris-HCl for 8 and glycine-NaOH for 9-11.

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## 계 골격근에서 15S ATPase의 순수분리 및 특성연구

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계골격근에서 일반적인 column chromatography와 glycerol density gradient centrifugation을 통하여, 한 ATPase를 순수하게 분리하였다. 이 효소는 gradient centrifugation 결과 15S의 침강계수를 갖기 때문에, 15S ATPase라고 부르게 되었다. Superose-6 column을 이용한 gel filtration analysis로부터 이 효소가 600-kDa의 크기를 갖는 분자인 것을 알 수 있었다. 그러나, sodium dodecyl sulfate 존재 하에서 polyacrylamide gel 전기영동을 수행하였을 때, 이 ATPase는 95-kDa의 폴리펩티드로 분리되었다. 따라서, 이 15S ATPase는 여섯개의 95-kDa 단위체로 구성되어 있음을 알 수 있었다. 또한, 이 효소는 ATP에 대한 Km이 0.6 mM이었고, pH 9에서 최고의 활성도를 보였다.