PROPERTIES OF MUSCLE REGULATORY PROTEINS FROM CHICKEN GIZZARD

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CONTENTS

PART I

Isolation and localization of a chicken gizzard inhibitory protein of Mg^{++}-activated skeletal muscle actomyosin ATPase

INTRODUCTION

Regulatory mechanism of vertebrate smooth muscle contraction has extensively been studied for recent years, and two types of regulatory system have been proposed. One is a myosin-linked system such as discovered in molluscan muscle and it functions through the phosphorylation of myosin by a Ca^{++}-dependent kinase (Bremel, 1974; Mrwa & Rüegg, 1975; Sobieszek & Bremel, 1975; Freason et al., 1976; Sobieszek & Small, 1977). The other is an actin-linked system which seems to be analogous to the troponin system in skeletal muscle in spite of the dissimilarity in molecular identity (Carsten, 1971; Sparrow & Bockxmeer, 1972; Ebashi et al., 1975; Ito & Hotta, 1976). Reconciliation of these two proposals seems to be very difficult and no attempt which relates the one type with the other has been reported yet.

On the other hand, a protein or protein complex analogous to troponin of skeletal muscle has been found in slime mold (Kato & Tonomura, 1975), platelet (Cohen et al., 1973), brain (Puszkin & Kochwa, 1974). Considering these reports altogether, one can suppose that troponin-like proteins play an important role in cell motility by regulating Ca^{++} concentration in cytoplasm. For the regulation, at least two types of regulatory proteins like troponin I and troponin C in skeletal muscle are considered to be necessary in any motile systems. However, there is rather few report on troponin I-like protein

while evidences to show the presence of Ca^{++}-binding protein like troponin C have been increasing.

We report in the present paper that an inhibitory protein of Mg^{++}-activated actomyosin ATPase (Mg^{++}-ATPase) could be isolated from frozen chicken gizzard muscle. This protein was of gizzard myofibrilar origin and the inhibitory activity of this protein on Mg^{++}-activated rabbit skeletal muscle actomyosin ATPase could be relieved by an addition of skeletal muscle troponin C.

MATERIALS AND METHODS

Preparation of a crude inhibitory protein from chicken gizzard

A crude inhibitory protein was prepared by the method of Ebashi et al. (1971) with a slight modification (Hirabayashi & Perry, 1974) from frozen or unfrozen chicken gizzard purchased directly from a slaughter house. Fresh unfrozen gizzard was also obtained from the chickens sacrificed just before the experimentation. In the final step of preparation the sample solution was dialyzed against 0.3mM $NaHCO_3$ containing 0.1mM $CaCl_2$ and freeze-dried. Detailed techniques for further purification of this protein will be described in the RESULTS section.

Preparation of gizzard myofibrils and thin filaments

Chicken gizzard myofibrils and thin filaments were prepared according to the procedure by Driska & Hartshorne (1975).

Preparation of skeletal muscle proteins from rabbits

Actin and myosin were prepared according to the methods described by Hirabayashi & Hayashi (1970) and by Perry (1955), respectively, and stored in 50% glycerol at -20°C. After

removal of glycerol by centrifugation or dialysis, actin and myosin were combined at a weight ratio of 1:4 to make reconstituted actomyosin which was used for the measurement of Mg^{++}-ATPase activity.

Chicken gizzard tropomyosin and rabbit skeletal muscle troponin were prepared according to the procedures based on the methods of Bailey (1948) and Ebahsi et al. (1971), respectively. Troponin C was obtained by the preparative electrophoresis using 5mm- or 10mm-thick slab gels as described elsewhere (Hirabayashi & Perry, 1974).

ATPase assays

The Mg^{++}-ATPase activity was determined 5 min after incubating the reaction medium at 25°C. The reaction medium contained 400-600µg of the reconstituted rabbit skeletal muscle actomyosin, 2.5mM $MgCl_2$, 2.5mM Tris (hydroxymethyl) aminomethane (Tris) ATP, and 25mM Tris-HCl (pH 7.6) in a total volume of 2ml. Either 1mM ethylene glycol bis-(2-amino-ethyl) tetraacetic acid (EGTA) or 0.05mM $CaCl_2$ was included in the medium to test Ca^{++}-sensitivity. The reaction was started by an addition of ATP and stopped by mixing the medium with 1ml 15% trichloroacetic acid.

Protein measurements

Protein concentration was determined by the procedure after Lowry *et al.* (1951) and by a micro method of N determination which involved nesslerization (Strauch, 1965).

Electrophoresis

Preparative slab-type electrophoresis in 10% (w/v) polyacrylamide gel was carried out by using a continuous buffer system of 25mM Tris, 80mM glycine, and 8M urea, pH 8.6 (Hirabayashi & Perry, 1974). Electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS) was performed by the method of Weber & Osborn (1969) in 0.1M sodium phosphate buffer (pH 7.0).

Chromatography

Diethylaminoethyl (DEAE)-Sephadex (A-50, Pharmacia) was equilibrated at 20°C with 20mM Tris-HCl buffer (pH 7.5) containing 6M urea and 15mM 2-mercaptoethanol, and packed into a glass column (1.7cm x 42cm). Crude inhibitory protein (60mg-100mg) from chicken gizzard was applied to the column and eluted by a linear gradient of 0-0.6M KCl. Fractions (4ml) were collected at a flow rate of 16ml/h. The fractions were dialyzed against distilled water, and freeze-dried.

Gel filtration

Gel filtration on Sephadex G-100 (Pharmacia) equilibrated with the Tris-HCl buffer as used for DEAE-Sephadex was carried out at 20°C on a column (2.5cm x 75cm) in which Sephadex G-100 was packed under the gravity. About 15mg of fraction I protein (Fig. 3) in 2ml of the Tris-HCl buffer was applied to the column and eluted at a flow rate of 12ml/h. 4ml fractions were collected, and those of protein peaks monitored by E_{280} measurement (Fig. 6) were pooled.

Immunological techniques

Antisera against the inhibitory protein were prepared in rabbits by injecting gel pieces containing the inhibitory protein band on SDS-electrophoresis. By cutting out the band most of small contamination was removed from peak I preparation after Sephadex G-100 gel filtration. Immunodiffusion tests were performed as described previously (Hirabayashi & Perry, 1974). Fluorescent antibody method (indirect method) was also carried out by the procedure reported previously (Hirabayashi & Hayashi, 1971).

RESULTS

Preparation of inhibitory protein

A protein fraction was prepared from frozen chicken gizzard according to the procedure of Ebashi et al. (1971) for skeletal muscle troponin preparation, and hereafter this fraction shall be referred to as "crude inhibitory protein" because of its inhibitory activity on the Mg^{++}-ATPase. About 25mg of the crude inhibitory protein was obtained from 100g of frozen gizzard muscle, and revealed to contain one major band, three or four minor bands and several minute bands on SDS-polyacrylamide gel electrophoresis (Fig. 1a). This protein fraction was also prepared from fresh unfrozen gizzard (Fig. 2a). Difference between the electrophoretic patterns of two protein fractions from frozen and unfrozen gizzards was not so obvious. Only the density of some minor bands was somewhat different. However, the effects of these protein fractions on the activity of Mg^{++}-ATPase of rabbit skeletal muscle were quite different (Fig. 1b and 2b). Every preparation from frozen gizzard showed remarkable inhibitory activity when 200μg of the protein was added to 500μg of the reconstituted actomyosin, while that from unfrozen fresh gizzard showed no inhibitory activity. It rather activated the ATPase at that concentration.

In order to identify the inhibitory component in the

preparation, further fractionation of the preparation from frozen gizzard was carried out by column chromatography on DEAE-Sephadex A-50. All protein, absorbed and non-absorbed, was divided into 6 fractions as shown in Fig. 3. Measurement of the inhibitory activity of the six fractions on the Mg^{++}-ATPase revealed that fractions I and II had the activity, while fractions III, IV, V, and VI had not (Fig. 4).

On SDS-polyacrylamide gel electrophoresis fraction I was found to contain two main protein bands of apparent molecular weights of 40,000 daltons and 19,000 daltons, and fraction II one major protein band of 23,000 daltons and one minor band of 40,000 daltons, while four fractions other than fractions I and II did not contain 40,000-dalton protein (Fig. 5). When the specific inhibitory activity of fractions I and II obtained in several trials of DEAE-Sephadex chromatography was examined in relation to the contents of 19,000-dalton and 40,000-dalton proteins, it has become clear that the inhibitory activity of each fraction was proportional to the content of the latter protein. Therefore, we concluded that 40,000-dalton protein was the inhibitory protein.

As the purity of the inhibitory protein in fraction I was only 30%, further purification was carried out by gel filtration on Sephadex G-100, and the elution profile is shown in Fig. 6a, where two peaks, I and II, were separated incompletely. Content of inhibitory protein in peak I was higher than in peak II which contained contaminations of

smaller-molecular-weight materials. On the average, about 3mg of inhibitory protein could be recovered from the peak I when 100g of frozen chicken gizzard muscle was used as a starting material.

Effect of troponin C and tropomyosin on the activity of the inhibitory protein

The inhibition of the Mg^{++}-ATPase of rabbit skeletal muscle actomyosin by the inhibitory protein was neutralized by an addition of rabbit skeletal muscle troponin C in the presence of 0.05mM $CaCl_2$, but the troponin C exerted no influence upon the inhibited ATPase activity under the presence of 1mM EGTA (Fig. 7). The skeletal muscle troponin C seemed to be as effective to inhibitory protein from gizzard as to the troponin I from skeletal muscle, whereas the effect of tropomyosin on the inhibitory protein was different from that on skeletal muscle troponin I, that is, even in the absence of tropomyosin the inhibition of the Mg^{++}-ATPase by the inhibitory protein was maintained (Fig. 8).

Localization of the inhibitory protein in gizzard

Localization of the inhibitory protein was examined by immunological techniques. The anti-inhibitory protein serum prepared by injecting the polyacrylamide gel containing

the inhibitory protein after electrophoresis into rabbits was specific for the antigen and formed only one precipitin line in an agar diffusion test by reacting with the crude inhibitory protein before column chromatography (Fig. 9). Specificity of the antiserum was also demonstrated by another method as follows.

The crude inhibitory protein was electrophoresed in SDS-polyacrylamide gel, and the gel was cut at every 3mm from the top to the bottom. Each gel piece was put in the order into the peripheral well of an agar plate to react with the antiserum charged in the central well. Only the gel pieces of Nos. 20, 21 and 22, which corresponded to the electrophoretic position of the inhibitory protein, formed a continuous precipitin line reacting with the antiserum (Fig. 10).

With this antiserum the gizzard muscle tissue was stained by an indirect fluorescent antibody method. The anti-inhibitory protein serum absorbed with the antigen was used as a control. As shown in Fig. 11, stained area was restricted to the muscle tissue just under the connective tissue. A stained area similar to this was also observed with anti-gizzard tropomyosin serum (Fig. 12), the specificity of which had been studied extensively in our laboratory (Hayashi et al., 1977).

In order to make sure that the inhibitory protein is one of the constituents of gizzard myofibrils, the antiserum was reacted in an agar plate with myofibrils, natural actomyosin

and thin filaments prepared from chicken gizzards, and formed, reacting with all these, a precipitin line totally identical with the one between the antiserum and the inhibitory protein. A typical example, in which the reaction of the antiserum with the thin filaments is observable, is shown in Fig. 13. However, the antiserum was reacted neither with troponin components from rabbit skeletal muscle nor with chicken gizzard tropomyosin.

DISCUSSION

The inhibitory protein of the Mg^{++}-ATPase was accidentally found in the protein preparation from chicken gizzard obtained by the procedure for obtaining the troponin from skeletal muscle after Ebashi et al. (1971). After several trials of obtaining the protein preparations it was found that only those from frozen gizzard were effective to inhibit the Mg^{++}-ATPase. We could obtain a preparation without inhibitory activity similar to those obtained from unfrozen gizzard when a mixture of frozen and unfrozen gizzard muscles was used as the starting materials. This fact suggests that the activity of protease which digests the constituent of smooth muscle myofibrils might be rather high in the unfrozen muscle extract. Therefore, we decided to prepare the protein preparation from frozen gizzard purchased from a slaughter house. It is highly possible that freezing the muscle tissue might cause some deterioration of its structural proteins, but its inhibitory activity seemed to be kept intact even when the tissue was frozen.

There is increasing evidence to show that troponin C or troponin C-like protein with Ca^{++}-binding ability exists in many organs other than skeletal muscle (Wolff & Siegel, 1972; Cohen et al., 1973; Childers & Siegel, 1975; Kuo & Coffee, 1976). However, successful isolation of troponin I or troponin I-like protein from non-skeletal muscle tissues

has not been reported yet. Mahendran & Berl (1977) reported that a component supposed to be troponin I-like protein was found in electrophoretic patterns of troponin-like fraction from bovine brain cortex. When the electrophoresis of skeletal muscle extract is conducted in the presence of Ca^{++} the complex of troponin C and troponin I migrates with about a half mobility of that of the troponin C into an alkaline-urea polyacrylamide gel. In other words, the retarded position of the troponin C in the presence of Ca^{++} suggests the existence of troponin I in the sample solution. According to the report by Head et al. (1977) troponin C-like protein in gizzard muscle extract migrated in the absence of Ca^{++}, but not in the presence. On the analogy of skeletal muscle extract, this behaviour of gizzard troponin C-like protein suggested the existence of a highly basic troponin I-like protein like the inhibitory protein reported in this paper.

We found troponin C-like and troponin I-like proteins in gizzard muscle. The biological function of the inhibitory protein in smooth muscle is somewhat uncertain. Although the inhibition of the Mg^{++}-ATPase activity by the inhibitory protein could be relieved by skeletal muscle troponin C, the molar ratio of the inhibitory protein to the troponin C for the effective relief, calculated from the result of Fig. 7, was very low if compared with that for skeletal muscle troponin I to troponin C (Perry et al., 1973). The protein does not need tropomyosin for its inhibitory activity unlike

the case of skeletal muscle troponin (Wilkinson et al., 1972). No component corresponding to the present inhibitory protein in molecular weight is found in the troponin or troponin-like fraction which has been reported to be fully functional in its regulation of the ATPase activity (Ebashi et al., 1975).

The experiment with anti-inhibitory protein serum revealed an important fact that the inhibitory protein is localized on thin filaments and in the smooth muscle tissue in more or less the same area as tropomyosin is. If it had been found to be localized otherwise, for example in nuclei, the protein might have been one of the basic proteins in nuclei. A basic protein in general is known to have an inhibitory effect on the Mg^{++}-ATPase (Syska et al., 1976). Localization of the inhibitory protein on thin filaments strongly suggests a possible role of the protein in the regulation of smooth muscle contraction.

In this experiment, reconstituted rabbit skeletal muscle actomyosin was used as the next-best alternative to measure the inhibitory activity of the troponin-like preparations from frozen chicken gizzard muscle, because gizzard actomyosin of no Ca^{++}-sensitivity was rather difficult to prepare in our laboratory. However, this combination of heterogeneous source materials might possibly lead to the false conclusion. A natural combination of gizzard inhibitory protein, troponin C-like protein, actomyosin and tropomyosin is preferable for the further investigation of the proteins by enzymological

methods.

SUMMARY

1. An inhibitory protein of Mg^{++}-activated actomyosin ATPase from rabbit skeletal muscle was prepared from frozen chicken gizzard and purified by DEAE-Sephadex chromatography and gel filtration.

2. The inhibition by this protein was relieved by an addition of skeletal muscle troponin C and was independent of gizzard tropomyosin.

3. Localization of the inhibitory protein in gizzard muscle tissue and gizzard thin filaments was demonstrated by immunohistological techniques and immunodiffusion tests.

PART II

Characterization of the inhibitory protein and troponin C-like protein from chicken gizzard

INTRODUCTION

In the previous section we reported the isolation and localization of the chicken gizzard inhibitory protein of the Mg^{++}-activated actomyosin ATPase (Mg^{++}-ATPase) and showed that the inhibitory protein has an apparent molecular weight of about 40,000 daltons and inhibits the Mg^{++}-ATPase of reconstituted actomyosin from rabbit skeletal muscle regardless of the presence or absence of Ca^{++}, and that the inhibition by the inhibitory protein is hardly potentiated by tropomyosin and can be neutralized by troponin C from skeletal muscle in the presence of Ca^{++}. Furthermore, it has been shown by using an antiserum against the inhibitory protein that the inhibitory protein exhibits similar localization to that of tropomyosin in chicken gizzard muscle. Thus the inhibitory protein has similar characteristics, if not identical, to those of troponin I from skeletal muscle in some respects.

There have been many reports on the regulatory mechanism of contraction in smooth muscle. Ebashi et al. (1966) prepared native tropomyosin from chicken gizzard muscle. They observed that the treatment of gizzard actomyosin by trypsin resulted in abolishment of its sensitivity to Ca^{++} and that the sensitivity was restored by adding the native tropomyosin.

Carsten (1971) studied on the preparation of tropomyosin-free troponin from uterine muscle. This protein showed at

least six bands on electrophoresis in the presence of sodium dodecyl sulfate (SDS). The major four bands of protein having molecular weights of approximately 14,500, 26,000, 43,000, and 56,000 daltons. Ca^{++}-sensitivity was restored to both desensitized skeletal and uterine actomyosin in the presence of the protein combined with tropomyosin from either uterus or skeletal muscle.

Driska & Hartshorne (1975) studied on the regulatory proteins in gizzard actomyosin purified by washing myofibrils with Triton X-100. They suggested that the ATPase activity of gizzard actomyosin is regulated by a protein of the thin filaments with a subunit weight of about 130,000 daltons. Recently, Mikawa *et al.* (1977) have reported that "gizzard troponin" obtained from native tropomyosin, having an apparent molecular weight of 80,000 daltons, could fully sensitize the desensitized actomyosin and tropomyosin alone had no such an effect.

On the other hand, Bremel (1974) presented evidence which supported the presence of the myosin-linked regulatory system in smooth muscle. He employed actin filaments without attached regulatory proteins to demonstrate the presence of a myosin-linked regulatory system in gizzard actomyosin. The Mg^{++}-ATPase showed the same sensitivity to Ca^{++} in the presence or absence of the added actin. Since the actin filaments contained no regulatory system the sensitivity to Ca^{++} must be caused by the myosin.

Very recently Small & Sobieszek (1977) have reported from a series of experiments that the triggering of the interaction between actin and myosin is mediated via a Ca^{++}-dependent phosphorylation of the 20,000 dalton-light chain of myosin which is in turn dependent on a specific myosin-light chain kinase, and that the dephosphorylation of the 20,000 dalton-light chain of myosin brought about by a phosphatase in the absence of Ca^{++} is associated with the inhibition or relaxation of the actin-myosin interaction.

As described above, several reports have suggested that there exists thick filament regulation involving a direct action of Ca^{++} on myosin, whereas others have found evidence for a tropomyosin-troponin-dependent regulatory system having properties different from that found in thin filament-regulated skeletal muscle. At present, the regulatory system of smooth muscle contraction has not been settled.

On the other hand, by the electrophoretic technique Perry et al. (1973) have demonstrated that skeletal muscle troponin I forms a Ca^{++}-dependent complex with troponin C, which is stable in 6M urea. Head et al. (1977) have shown by the same technique the presence of troponin C-like protein in chicken gizzard as well as in rabbit uterus and cow carotid.

In the present study, we compared some properties of the inhibitory protein with those of troponin I from skeletal muscle. In addition we confirmed the presence of troponin C-like protein in this tissue as Head et al. did. The protein

isolated by preparative polyacrylamide gel electrophoresis was compared with skeletal muscle troponin C enzymatically and electrophoretically, and the interaction of the troponin C-like protein with the inhibitory protein was investigated.

MATERIALS AND METHODS

Preparations of the inhibitory protein, rabbit skeletal muscle actin, myosin and troponin C

These were carried out by the same procedures as those described in PART I (Makioka & Hirabayashi, in preparation).

Preparation of troponin I and troponin T from rabbit skeletal muscle

Both troponin I and troponin T were isolated from the troponin complex by preparative polyacrylamide gel electrophoresis in 0.9M acetic acid buffer (pH 3.2) containing 8M urea.

Preparation of gizzard actomyosin

This was carried out as described by Driska & Hartshorne (1975) and desensitization of natural actomyosin was conducted as described by Sparrow & Bockxmeer (1972).

Electrophoresis

Polyacrylamide gel electrophoresis was carried out under the same conditions as described in PART I.

Muscle extract for electrophoresis was prepared as follows. Frozen or unfrozen muscle was excised in about 4 vol of an 8M urea solution containing 50mM 2-mercaptoethanol. The suspension of the excised muscle was then dialyzed for at least 4 hr against the same solution and centrifuged at 27,000xg for 30 min to remove insoluble material, and the supernatant was used for electrophoresis. In order to detect the change of electrophoretic pattern due to coexisting Ca^{++} ion, 5mM ethylene glycol bis-(2-aminoethyl) tetraacetic acid (EGTA) was added to the sample solution for Ca^{++}-free extract and 5mM $CaCl_2$ was added for Ca^{++}-containing extract. When non-dissociating conditions of protein components were required the urea was replaced by 40% (v/v) glycerol (Perrie & Perry, 1970).

Interaction of the inhibitory protein with F-actin

The inhibitory protein was mixed with F-actin in a solution containing 0.1M KCl, 2mM $MgCl_2$ and 10mM imidazole (pH 7.0) and centrifuged at 100,000xg for 2 hr at 4°C. The pellets were suspended in 0.01M phosphate buffer (pH 7.0) containing 4% SDS and dialyzed against the buffer. The supernatants were used for the determination of protein.

ATPase assays for gizzard actomyosin

ATPase activity was measured by incubating the actomyosin at 35°C for 30 min in 10mM $MgCl_2$, 2.5mM Tris-ATP, and 25mM Tris (pH 7.6).

RESULTS

(I) Comparison of the inhibitory activity of the inhibitory protein with that of troponin I from skeletal muscle

The inhibitory activity of the inhibitory protein on the Mg^{++}-ATPase of reconstituted actomyosin from rabbit skeletal muscle was higher than that of troponin I in the absence of tropomyosin (Fig. 14). In the presence of tropomyosin, the amount of the inhibitory protein required for 50% inhibition of the ATPase activity was almost equal to that of troponin I (Fig. 15). Thus, the inhibitory activity of the inhibitory protein was almost independent of tropomyosin.

Binding of the inhibitory protein to rabbit skeletal muscle actin

It has been established that troponin I from skeletal muscle prevents the interaction of actin with myosin via a troponin I-actin interaction, which decreases the rate of hydrolysis of Mg-ATP (Perry et al., 1973). Therefore, it is interesting to compare the interaction of the inhibitory protein with F-actin with that of troponin I.

Various amounts (37-444µg) of the inhibitory protein were mixed with F-actin (0.99mg) in a 0.1M KCl solution

and the inhibitory protein precipitated with F-actin was analyzed by SDS-polyacrylamide gel electrophoresis. The inhibitory protein without F-actin was not precipitated by the centrifugation under the same condition. As shown in Fig. 16, about 2mol of the inhibitory protein were bound to 7mol of actin. On the other hand, 0.6mol of troponin I from skeletal muscle has been known to bind to 7mol of actin when no tropomyosin is present (Potter & Gergely, 1974).

Effect of the inhibitory protein on the Mg^{++}-ATPase of desensitized actomyosin from chicken gizzard muscle

Natural actomyosin was prepared from frozen chicken gizzard muscle by the method of Driska & Hartshorne (1975), and the Mg^{++}-ATPase was assayed in the presence and absence of Ca^{++}. As shown in Fig. 17, the Mg^{++}-ATPase activity in the presence of 3mM EGTA was at most 50% of the activity in the absence of EGTA.

To desensitize the gizzard actomyosin the procedure described by Sparrow & Bockxmeer for arterial actomyosin (1972) was employed. However, the Mg^{++}-ATPase activity of the actomyosin prepared by their procedure was affected by changes in the Ca^{++} concentration, i.e., Ca^{++}-sensitivity still remained. The effect of the inhibitory protein on the Mg^{++}-ATPase activity was investigated with this actomyosin, although desensitized incompletely. Fig. 18 shows that the

inhibitory protein inhibited the Mg^{++}-ATPase activity of gizzard actomyosin in the presence of Ca^{++}, but that at most 50% of the ATPase activity in the absence of the inhibitory protein was inhibited in the presence of the protein.

(II) Evidence for the existence of troponin C-like protein in chicken gizzard muscle

Alkaline-urea-polyacrylamide gel electrophoresis was carried out to detect troponin C-like protein in chicken gizzard. The electrophoretic pattern of muscle extract from frozen chicken gizzard in 25mM Tris-80mM glycine buffer (pH 8.6) containing 8M urea, was compared in the presence and absence of Ca^{++}. In the absence of Ca^{++}, two fast moving bands could be detected (Fig. 19). In the presence of Ca^{++}, the faster band of the two disappeared while the slower remained. This electrophoretic pattern suggested that troponin C-like protein formed a complex with a certain protein, probably a troponin I-like protein in the presence of Ca^{++}, since under the same conditions skeletal muscle troponin C formed a complex with skeletal muscle troponin I. The disappearance of the faster band in the presence of Ca^{++} was observed only when frozen gizzard was used as material for the protein preparation. If fresh unfrozen gizzard was used and the extract was electrophoresed in the same condition as mentioned above, the band pattern was significantly

different from that in the case of frozen muscle extract (Fig. 20). On removal of Ca^{++} the band of the troponin C-like protein was conspicuously detectable, and it did not disappear completely even in the presence of Ca^{++}, and other faster migrating bands than that of the troponin C-like protein were found unlike the case with frozen muscle extract.

To confirm the existence of the complex of troponin C-like protein with troponin I-like protein in the presence of Ca^{++}, two-dimensional gel electrophoresis was conducted by using the same buffer system as mentioned above. After muscle extract of frozen chicken gizzard was electrophoresed in the presence of Ca^{++}, the slab gel was cut vertically and laid on the second slab gel horizontally, and the second electrophoresis was carried out with the addition of a 0.1M EGTA solution onto the top of the gel strips. As shown in Fig. 21, when Ca^{++} was present in dimension one, a fast moving band (arrow) appeared by the addition of EGTA in the position corresponding to the top of gel in the first electrophoresis.

On the other hand, when EGTA was present in dimension one, any band corresponding to the troponin C-like protein did not appear in dimension two. From these results it can be said that the troponin I-like protein might be of a strongly basic nature, and that the complex stays at or close to the origin on polyacrylamide gel and does not migrate

into the gel.

When urea was replaced with 40% (v/v) glycerol, in the absence of Ca^{++} the troponin C-like protein migrated as a single band to the anode more rapidly than any other proteins (Fig. 22), and the formation of the complex was observed in the presence of Ca^{++} as in the case with the urea gel. Under the same conditions, the troponin C-like protein could be isolated by preparative polyacrylamide gel electrophoresis. The protein obtained migrated as a single band and its apparent molecular weight was calculated to be about 15,500 daltons on electrophoresis in 0.1M sodium phosphate buffer (pH 7.0) containing 0.1% SDS (Fig. 23).

Effect of the troponin C-like protein on the Mg^{++}-ATPase of rabbit skeletal muscle actomyosin

To investigate the functional characteristics of the troponin C-like protein it was tested whether the troponin C-like protein functions like troponin C from skeletal muscle in an ATPase assay system with reconstituted actomyosin from rabbit skeletal muscle. In the presence of gizzard tropomyosin and rabbit troponin I the troponin C-like protein was found to function in a very similar manner to that of skeletal muscle troponin C, relieving the inhibition by troponin I of the Mg^{++}-ATPase, both in the presence and absence of Ca^{++} (Fig. 24). Relief of the inhibition was completed when the

molar ratio of the troponin C-like protein to troponin I was about 3. The troponin C-like protein alone had no effect on the Mg^{++}-ATPase either in the presence or absence of Ca^{++}.

When troponin T was added to the assay system, the troponin C-like protein could not confer Ca^{++}-sensitivity on the ATPase (Fig. 25) while skeletal muscle troponin C could.

Comparison of the electrophoretic mobility of the troponin C-like protein with that of skeletal muscle troponin C in the presence or absence of urea

On alkaline-urea-polyacrylamide gel electrophoresis at pH 8.6, the electrophoretic mobility of the troponin C-like protein was slightly reduced in the absence of Ca^{++} just as that of troponin C from rabbit skeletal muscle was (Fig. 26). On the other hand, by the replacement of urea by 40% glycerol in the electrophoretic conditions, the mobility of the troponin C-like protein was drastically changed depending on the concentration of Ca^{++}: the mobility was significantly reduced in the presence of Ca^{++} unlike the increase in mobility on the urea gel; whereas skeletal muscle troponin C migrated faster in the presence of Ca^{++} than in the absence of Ca^{++} (Fig. 27).

(III) Complex formation of the troponin C-like protein with troponin I or troponin T from skeletal muscle

At pH 8.6, in the absence of urea, the troponin C-like protein formed a complex with troponin I from skeletal muscle in the presence of Ca^{++} (Fig. 28). On electrophoresis the complex moved with a mobility of about 30% of that of the troponin C-like protein. The complex was not formed in the absence of Ca^{++}. In the presence of urea, the complex was also formed, but which stayed at the origin on polyacrylamide gel and did not migrate with an intermediate mobility between those of the two proteins unlike the case on the gel where urea was absent (Data were not shown).

Likewise the troponin C-like protein formed a complex with troponin T from skeletal muscle. The complex moved more slowly than the complex of the troponin C-like protein and troponin I, and the mobility was about 14% of that of the troponin C-like protein (Fig. 29).

Interaction of the inhibitory protein with the troponin C-like protein from chicken gizzard muscle

When a mixture of the inhibitory protein and the troponin C-like protein was electrophoresed on alkaline polyacrylamide gel in the presence of Ca^{++}, a band of the complex was found, and the mobility was about 11% of that of the troponin C-like

protein alone (Fig. 30). Under these conditions the inhibitory protein in the absence of the troponin C-like protein did not move towards the anode. This strongly suggests that the inhibitory protein can form a Ca^{++}-dependent complex with the troponin C-like protein as skeletal muscle troponin I does with skeletal muscle troponin C.

To study the effect of the troponin C-like protein on the inhibition of the Mg^{++}-ATPase of reconstituted actomyosin from rabbit skeletal muscle by the inhibitory protein, the troponin C-like protein was added to the Mg^{++}-ATPase assay system containing reconstituted actomyosin, gizzard tropomyosin and the inhibitory protein. The result shown in Fig. 31 indicates that the troponin C-like protein can relieve the inhibition induced by the inhibitory protein, both in the presence and absence of Ca^{++}, in other words, the relief is not Ca^{++}-sensitive.

DISCUSSION

Since the inhibition of the Mg^{++}-ATPase by the chicken gizzard inhibitory protein is not affected by the presence of tropomyosin, the mechanism of inhibition might be qualitatively different from that in skeletal muscle where the activity is potentiated by tropomyosin (Figs. 14 and 15). The inhibitory activity of the inhibitory protein can be distinguished from the non-specific inhibitory activity of basic proteins such as salmine, lysozyme and cytochrome C since the inhibition of such basic proteins is neutralized by the addition of tropomyosin (Syska et al., 1976).

Although maximum inhibition induced by the inhibitory protein is slightly lower than that by troponin I in the presence of gizzard tropomyosin, the amount of the inhibitory protein required for 50% inhibition is almost identical with that of troponin I.

Evidence has been shown that the specific inhibition of actomyosin ATPase by troponin I occurrs by interacting with actin by Perry et al. (1973). As for troponin I from rabbit skeletal muscle, it has been shown that the region containing residues 96-117 has a special affinity for actin, and is responsible for the inhibitory activity on the Mg^{++}-ATPase of actomyosin (Syska et al., 1976). The experiments by ultracentrifugation clearly indicate that the inhibitory protein binds to F-actin at a ratio of 2mol per 7 actin

monomers (Fig. 16), and presumably this interaction causes the inhibition of the Mg^{++}-ATPase.

The effect of the inhibitory protein of chicken gizzard origin was investigated on the Mg^{++}-ATPase of reconstituted actomyosin from rabbit skeletal muscle. It is necessary to examine whether the inhibitory protein shows the inhibitory activity on the Mg^{++}-ATPase of gizzard actomyosin. However, Ca^{++}-sensitivity of gizzard natural actomyosin we prepared was as high as 50%, and the desensitization of gizzard actomyosin has not been successful (Fig. 17). Even with such incompletely desensitized gizzard actomyosin, it has become clear that the inhibitory protein inhibits the Mg^{++}-ATPase of gizzard actomyosin in the presence of Ca^{++} (Fig. 18).

Therefore the inhibitory protein seems to be essentially similar to skeletal muscle troponin I in their ability to inhibit the actomyosin Mg^{++}-ATPase activity irrespective of Ca^{++} concentration. From the fact that troponin C is required to neutralize the inhibition by skeletal muscle troponin I, it is very natural to expect that the inhibitory protein might need a certain protein corresponding to skeletal muscle troponin C. In order to detect troponin C or troponin C-like protein, the technique by Perry *et al.* (1973), with which Ca^{++}-dependent troponin I-troponin C complex was demonstrated, was employed. Head *et al.* (1977) have already demonstrated the presence of troponin C-like protein in many smooth muscles including chicken gizzard. However, they have not detected

the opponent of the troponin C-like protein, i.e., troponin I-like protein. Since the troponin C-like proteins have been isolated not only from muscle tissues but also from many other non-muscle tissues such as brain (Fine et al., 1975), adrenal medulla (Kuo & Coffee, 1976) and platelet (Muszbek et al., 1977), and successful isolation of troponin I-like protein has not so far reported, troponin C-like protein seems to be prepared more easily than troponin I-like protein.

In the present work, we detected the troponin C-like protein in chicken gizzard (Fig. 19) confirming the results of Head et al. (1977). Furthermore, we found that the electrophoretic pattern of gizzard muscle was significantly different depending on whether the muscle was frozen or unfrozen (Figs. 19 and 20). The band corresponding to the troponin C-like protein did not disappear completely in the presence of Ca^{++} when the protein was extracted from unfrozen gizzard muscle, while it disappeared completely when the protein was extracted from frozen muscle. Probably the complex of troponin C-like protein and some basic protein might be unstable in 8M urea due to the presence of protease in the unfrozen muscle extract, and the possibility that some protein with same mobility as that of the troponin C-like protein is present in the muscle extract is not completely excluded yet.

The replacement of urea in the gel by 40% glycerol produced a significant change in the electrophoretic pattern

of muscle extract. Most of bands tended to become broader in the presence of glycerol. The troponin C-like protein moves well in front of the other bands. The isolation of the pure troponin C-like protein was therefore performed by polyacrylamide gel electrophoresis in the presence of glycerol instead of urea (Fig. 22).

The protein thus obtained was proved to move as a single band in SDS-polyacrylamide gel with a mobility corresponding to an apparent molecular weight of 15,500 daltons (Fig. 23), which is smaller than those of troponin C from skeletal muscle (18,500) (Hartshorne & Pyun, 1971; Schaub & Perry, 1971), troponin C-like proteins from brain (17,000) (Fine et al., 1975), adrenal medulla (16,000) (Kuo & Coffee, 1976) and platelet (16,500) (Muszbek et al., 1977), but larger than those of parvalbumins from vertebrates (11,000-13,000) (Pechére et al., 1971; Capony et al., 1976).

The combined evidence obtained in enzymic and electrophoretic studies suggests that our troponin C-like protein from chicken gizzard is similar to troponin C from skeletal muscle in several characteristics. For example, its mobility increases on the urea gel by the presence of Ca^{++} (Fig. 26), and it can form complexes with troponin I and troponin T from skeletal muscle (Figs. 28 and 29), which are Ca^{++}-dependent, and it can relieve the inhibition by troponin I on the Mg^{++}-ATPase both in the presence and absence of Ca^{++} (Fig. 23). In spite of these similarities, the troponin C-

like protein is different from troponin C from skeletal muscle in that the mobility of the protein on the glycerol gel is significantly reduced by an addition of Ca^{++} (Fig. 27), and that it does not confer Ca^{++}-sensitivity on the Mg^{++}-ATPase in the presence of tropomyosin, troponin I and troponin T (Fig. 25).

Since it is evident that the inhibitory protein can interact with the troponin C-like protein of chicken gizzard, forming the Ca^{++}-dependent complex, and that the troponin C-like protein and the inhibitory protein are capable of controlling the rabbit reconstituted actomyosin ATPase activity, the inhibitory protein we isolated is one of the most probable candidate for troponin I-like protein which control the Mg^{++}-ATPase activity of chicken gizzard actomyosin in collaboration with the troponin C-like protein. If such be the case, it is expected that the complex, troponin C-like and troponin I-like proteins, may participate in the regulation of smooth muscle contraction.

SUMMARY

1. Besides the inhibitory protein of Mg^{++}-activated actomyosin ATPase from rabbit skeletal muscle, a troponin C-like protein was prepared from frozen chicken gizzard, and the functional validity of the two proteins in the regulation of smooth muscle contraction was investigated by enzymological and electrophoretic techniques.

2. The amount of the inhibitory protein required for 50% inhibition of the Mg^{++}-activated actomyosin ATPase was almost equal to that of troponin I from skeletal muscle.

3. The inhibitory protein could inhibit the Mg^{++}-activated ATPase of partially desensitized gizzard actomyosin in the presence of Ca^{++}.

4. The inhibitory protein bound to F-actin from skeletal muscle at a physiological condition.

5. The chicken gizzard troponin C-like protein obtained by preparative polyacrylamide gel electrophoresis had an apparent molecular weight of 15,500 daltons.

6. On alkaline-urea gel, the mobility of the troponin C-like protein increased slightly in the presence of Ca^{++} as

that of skeletal muscle troponin C did. On the other hand, the mobility decreased significantly on alkaline-glycerol gel in the presence of Ca^{++} unlike the case with skeletal muscle troponin C.

7. The troponin C-like protein could form Ca^{++}-dependent complex with troponin I or troponin T from skeletal muscle.

8. The troponin C-like protein could neutralize the inhibition by skeletal muscle troponin I of the Mg^{++}-activated actomyosin ATPase from rabbit skeletal muscle, but could not confer Ca^{++}-sensitivity to the actomyosin in the presence of troponin I and troponin T from skeletal muscle.

9. The troponin C-like protein could form a Ca^{++}-dependent complex with the inhibitory protein and could neutralize the inhibition by the inhibitory protein of the Mg^{++}-activated actomyosin ATPase from rabbit skeletal muscle regardless of the presence or absence of Ca^{++}.

EXPANATION OF FIGURES

Fig. 1. The crude inhibitory protein prepared from frozen chicken gizzard muscle.

(a) Electrophoresis in 7.5% (w/v) polyacrylamide gel containing 0.1M sodium phosphate buffer (pH 7.0) and 0.1% sodium dodecyl sulfate. The 40,000-dalton protein band is indicated by an arrow. O, Origin; +, anode.

(b) Effect of the crude inhibitory protein from chicken gizzard muscle on the activity of Mg^{++}-activated actomyosin ATPase from rabbit skeletal muscle. ATPase assays were carried out under the standard conditions (see MATERIALS AND METHODS), with 580μg of reconstituted rabbit actomyosin in the presence of chicken gizzard tropomyosin (72μg). Activity is expressed as a percentage of that obtained in the absence of inhibitory protein and EGTA. ●, In the presence of 0.05mM $CaCl_2$; o, in the presence of 1mM EGTA.

Fig. 2. The crude protein from fresh (not frozen) chicken gizzard muscle.

(a) Electrophoresis in 7.5% (w/v) polyacrylamide gel containing 0.1M sodium phosphate buffer (pH 7.0) and 0.1% sodium dodecyl sulfate. Note the absence of 40,000-dalton protein band (cf. Fig. 1a).

(b) Effect of the crude protein from fresh chicken gizzard muscle on the activity of Mg^{++}-activated actomyosin ATPase

from rabbit skeletal muscle. ATPase assays were carried out under the standard conditions, with 608μg of reconstituted rabbit actomyosin in the presence of chicken gizzard tropomyosin (73μg). Activity is expressed as a percentage of that obtained in the absence of the crude protein and EGTA.
●, In the presence of 0.05mM $CaCl_2$; O, in the presence of 1mM EGTA.

Fig. 3. Chromatography of the crude inhibitory protein on DEAE-Sephadex A-50.

A sample (60mg) of crude inhibitory protein from frozen gizzard was applied to a column (1.7cm x 42cm) of DEAE-Sephadex A-50 equilibrated against 20mM Tris-HCl buffer (pH 7.5) containing 6M urea and 15mM 2-mercaptoethanol. A linear gradient of 0-0.6M KCl was applied after 120ml of the Tris-HCl buffer had been eluted. Every 4ml eluate was collected. Six fractions as shown on the upper part of the figure were pooled and used for electrophoretic analysis.

Fig. 4. Effect of fractions from DEAE-Sephadex chromatography on Mg^{++}-activated ATPase of reconstituted rabbit actomyosin.

ATPase assays were carried out under the standard conditions, with 448μg of reconstituted rabbit actomyosin in the presence of chicken gizzard tropomyosin (96μg). Activity is expressed as a percentage of that obtained in the absence of fractions from the chromatography and EGTA. Closed symbols,

in the presence of 0.05mM $CaCl_2$; open symbols, in the presence of 1mM EGTA.

(a) ●, ○, fraction I; ▲, △, fraction II; ■, □, fraction IV.

(b) ●, ○, fraction III; ▲, △, fraction V; ■, □, fraction VI.

Fig. 5. SDS-polyacrylamide gel electrophoresis of fractions from DEAE-Sephadex chromatography.

Electrophoresis was conducted in 7.5% (w/v) polyacrylamide gel containing 0.1M sodium phosphate buffer (pH 7.0) and 0.1% sodium dodecyl sulfate. The fraction numbers correspond to those in Fig. 3. Purity of 40,000-dalton protein in fraction I is about 30%.

Fig. 6. Gel filtration of fraction I on Sephadex G-100.

Fraction I (15mg of protein) from DEAE-Sephadex chromatography was dissolved in 20mM Tris-HCl buffer (pH 7.5) containing 6M urea and 15mM 2-mercaptoethanol, and applied to a column (2.5cm x 75cm) of Sephadex G-100 equilibrated against the buffer. Each 4ml eluate was collected (a) and the peaks I and II were analyzed by SDS-polyacrylamide gel electrophoresis (b).

Fig. 7. Effect of troponin C from rabbit skeletal muscle on the inhibitory state of Mg^{++}-activated ATPase of reconstituted rabbit actomyosin by the inhibitory protein.

ATPase assays were carried out under the standard

conditions, with 600μg of reconstituted rabbit actomyosin in the presence of chicken gizzard tropomyosin (73μg) and the inhibitory protein (18μg). Activity is expressed as a percentage of that obtained in the absence of troponin C and the inhibitory protein. ●, In the presence of 0.05mM $CaCl_2$; ○, in the presence of 1mM EGTA.

Fig. 8. Tropomyosin-dependency of the inhibitory activity of the inhibitory protein.

ATPase assays were carried out under the standard conditions, with 198μg of reconstituted rabbit actomyosin in the presence (●) or absence (○) of chicken gizzard tropomyosin (77μg). Increasing amounts of the inhibitory protein from DEAE-Sephadex chromatography were added. Activity is expressed as a percentage of that obtained in the absence of the inhibitory protein.

Fig. 9. Reactions of anti-inhibitory protein serum with the inhibitory protein in an agar diffusion test. anti-IP, rabbit anti-inhibitory protein serum; IP, the inhibitory protein purified by gel filtration; C, the crude inhibitory protein. IP and C were sequentially diluted as shown in the wells starting from concentration of 120μg/ml and 1mg/ml, respectively.

Fig. 10. Specificity of anti-inhibitory protein serum.

The crude inhibitory protein (180μg) from frozen gizzard was electrophoresed in SDS-7.5% (w/v) polyacrylamide gel. The gel was cut into slices of 3mm in thickness and put in the wells on the agar plate in the order as indicated on the figure. Only the gels of Nos. 20, 21 and 22 have formed a precipitin line reacting with the antiserum in the central well.

Fig. 11. Fluorescence micrographs of chicken gizzard muscle stained with anti-inhibitory protein serum (a) and with the antiserum absorbed with the inhibitory protein (b). The bars inserted indicate 40μ.

Fig. 12. Fluorescence micrographs of chicken gizzard muscle stained with anti-gizzard tropomyosin serum (a) and with the antiserum absorbed with the tropomyosin (b). The bars inserted indicate 40μ.

Fig. 13. Reaction of anti-inhibitory protein serum with the chicken gizzard thin filaments.
(a) SDS-polyacrylamide gel electrophoresis of chicken gizzard thin filaments used in (b).
(b) Immunoreaction of anti-inhibitory protein serum with the chicken gizzard thin filaments.
anti-IP, anti-inhibitory protein serum; ab.anti-IP, anti-inhibitory protein serum absorbed with the antigen; IP, inhibitory protein; GTF, chicken gizzard thin filaments.

Fig. 14. Comparison of the inhibitory activity of the inhibitory protein with that of skeletal muscle troponin I on the Mg^{++}-activated ATPase of reconstituted rabbit actomyosin in the absence of tropomyosin.

ATPase activity is presented as a percentage of that obtained in the absence of the inhibitory protein, rabbit troponin I and EGTA. Assays were carried out under the standard conditions, with 406µg of reconstituted rabbit actomyosin. Closed symbols, in the presence of 0.05mM $CaCl_2$; open symbols, in the presence of 1mM EGTA. ●, ○, Inhibitory protein; ▲, △, troponin I from rabbit skeletal muscle.

Fig. 15. Comparison of the inhibitory activity of the inhibitory protein with that of skeletal muscle troponin I on the Mg^{++}-activated ATPase of reconstituted rabbit actomyosin in the presence of tropomyosin.

ATPase activity is presented as a percentage of that obtained in the absence of the inhibitory protein, rabbit troponin I and EGTA. Assays were carried out under the standard conditions, with 384µg of reconstituted rabbit actomyosin and 91µg of gizzard tropomyosin. Closed symbols, in the presence of 0.05mM $CaCl_2$; open symbols, in the presence of 1mM EGTA. ●, ○, Inhibitory protein; ▲, △, troponin I from rabbit skeletal muscle.

Fig. 16. Interaction of the inhibitory protein with F-actin from skeletal muscle.

Inhibitory protein (0.3mg/ml) was mixed with F-actin (0.99mg/ml) in a solution containing 0.1M KCl, 10mM imidazole (pH 7.0) and 2mM $MgCl_2$. After centrifugation at 100,000xg for 2 hr at 4°C, the pellets were treated with SDS for electrophoresis as described under MATERIALS AND METHODS.

(a) F-actin

(b) F-actin + inhibitory protein

(c) Inhibitory protein

Fig. 17. Effect of EGTA on the Mg^{++}-activated ATPase of gizzard natural actomyosin.

ATPase activity is presented as a percentage of that obtained in the presence of 0.05mM $CaCl_2$. Assays were carried out in a solution of 2.5mM ATP, 10mM $MgCl_2$ and 25mM Tris (pH 7.6) containing 994µg of gizzard natural actomyosin.

Fig. 18. Effect of the inhibitory protein on the Mg^{++}-activated ATPase of desensitized gizzard actomyosin.

ATPase activity is presented as a percentage of that obtained in the absence of the inhibitory protein and EGTA. Assays were carried out under the same conditions as shown in Fig. 17, with 1.01mg of desensitized gizzard actomyosin. ●, In the presence of 0.05mM $CaCl_2$; O, in the presence of 1mM EGTA.

Fig. 19. Polyacrylamide gel electrophoresis of frozen-gizzard muscle extract in the presence or absence of $CaCl_2$.

Muscle extract was prepared in 4 vol of an 8M urea solution containing 50mM 2-mercaptoethanol as described in the MATERIALS AND METHODS and about 0.1ml sample was applied on the gel and electrophoresis was carried out in 10% polyacrylamide gel containing 25mM Tris-80mM glycine buffer (pH 8.6) and 8M urea. 5mM $CaCl_2$ (a) or 5mM EGTA (b) was added to the sample solution. Presumed troponin C-like protein is indicated by an arrow. O, Origin; +, anode.

Fig. 20. Polyacrylamide gel electrophoresis of unfrozen-gizzard muscle extract in the presence or absence of $CaCl_2$.

Conditions were as for Fig. 19. Unfrozen muscle extract was electrophoresed in the presence of 5mM $CaCl_2$ (a) or 5mM EGTA (b) in the sample solution. Presumed troponin C-like protein is indicated by an arrow. O, Origin; +, anode.

Fig. 21. Two-dimensional gel electrophoresis of gizzard muscle extract.

Muscle extract was made in 4 vol of an 8M urea solution containing 50mM 2-mercaptoethanol as described in the MATERIALS AND METHODS. 5mM EGTA (a) or 5mM $CaCl_2$ (b) was added to the sample solution. The first electrophoresis was carried out by applying about 0.1ml sample of muscle extract on the 10% polyacrylamide slab type gel containing 25mM Tris-80mM

glycine buffer (pH 8.6) and 8M urea. Each gel was cut vertically and laid on the second slab gel horizontally, and the second electrophoresis under the same conditions was carried out with the addition of a 0.1M EGTA solution onto the top of the gel strips.

Fig. 22. Polyacrylamide gel electrophoresis of gizzard muscle extract in the absence of urea.

Muscle extract was made in 4 vol of an 8M urea solution containing 50mM 2-mercaptoethanol as described in the MATERIALS AND METHODS and about 0.1ml sample of muscle extract was applied on the gel. The electrophoresis was carried out in 10% polyacrylamide gel containing 25mM Tris-80mM glycine buffer (pH 8.6) and 40% (v/v) glycerol. 5mM $CaCl_2$ (a) or 5mM EGTA (b) was added to the sample solution. Presumed troponin C-like protein is indicated by an arrow. 0, Origin; +, anode.

Fig. 23. Molecular weight determination of purified gizzard troponin C-like protein by SDS-polyacrylamide gel electrophoresis.

(a) Electrophoresis was carried out in 7.5% polyacrylamide gel containing 0.1M sodium phosphate buffer (pH 7.0) and 0.1% SDS. 0, Origin; +, anode. 33μg of protein was applied on the gel.

(b) Semilogarithmic plot of molecular weight against mobility

for proteins electrophoresed in 5% polyacrylamide gel containing 0.1M sodium phosphate buffer (pH 7.0) and 0.1% SDS. 1, Bovine serum albumin; 2, egg albumin; 3, pepsin; 4, lysozyme. Arrow indicates the position of gizzard troponin C-like protein.

Fig. 24. Effect of gizzard troponin C-like protein on the inhibition of the Mg^{++}-activated ATPase of reconstituted rabbit actomyosin by skeletal muscle troponin I.

ATPase activity is presented as a percentage of that obtained in the absence of rabbit troponin I, gizzard troponin C-like protein and EGTA. Assays were carried out under the standard conditions, with 397µg of reconstituted rabbit actomyosin, 81µg of gizzard tropomyosin and 33µg of rabbit troponin I. ●, In the presence of 0.05mM $CaCl_2$; ○, in the presence of 1mM EGTA.

Fig. 25. Effect of gizzard troponin C-like protein on the Mg^{++}-activated ATPase of reconstituted rabbit actomyosin in the presence of both troponin I and troponin T from skeletal muscle.

ATPase activity is presented as a percentage of that obtained in the absence of gizzard troponin C-like protein, troponin I, troponin T and EGTA. Assays were carried out under the standard conditions, with 397µg of reconstituted rabbit actomyosin, 81µg of gizzard tropomyosin, 33µg of rabbit troponin I, and 47µg of rabbit troponin T. ●, In the

presence of 0.05mM $CaCl_2$; O, in the presence of 1mM EGTA.

Fig. 26. Comparison of the electrophoretic mobility of gizzard troponin C-like protein with that of skeletal muscle troponin C in the presence of urea.

Electrophoresis was carried out under the same conditions as for Fig. 19. O, Origin; +, anode.

(a) 94μg of rabbit troponin C with 5mM $CaCl_2$

(b) as (a) but with 5mM EGTA in place of $CaCl_2$

(c) 33μg of gizzard troponin C-like protein with 5mM $CaCl_2$

(d) as (c) but with 5mM EGTA in place of $CaCl_2$

Fig. 27. Comparison of the electrophoretic mobility of gizzard troponin C-like protein with that of skeletal muscle troponin C in the absence of urea.

Electrophoresis was carried out under the same conditions as for Fig. 22. O, Origin; +, anode.

(a) 100μg of rabbit troponin C with 5mM $CaCl_2$

(b) as (a) but with 5mM EGTA in place of $CaCl_2$

(c) 33μg of gizzard troponin C-like protein with 5mM $CaCl_2$

(d) as (c) but with 5mM EGTA in place of $CaCl_2$

Fig. 28. Complex formation of gizzard troponin C-like protein with skeletal muscle troponin I.

Electrophoresis was carried out under the same conditions as for Fig. 22. The band of the complex is indicated by

an arrow. O, Origin; +, anode.

(a) 50μg of gizzard troponin C-like protein + 50μg of rabbit troponin I + 5mM $CaCl_2$

(b) as (a) but with 5mM EGTA in place of $CaCl_2$

Fig. 29. Complex formation of gizzard troponin C-like protein with skeletal muscle troponin T.

Electrophoresis was carried out under the same conditions as for Fig. 22. The band of the complex is indicated by an arrow. O, Origin; +, anode.

(a) 50μg of gizzard troponin C-like protein + 50μg of rabbit troponin T + 5mM $CaCl_2$

(b) as (a) but with 5mM EGTA in place of $CaCl_2$

Fig. 30. Complex formation of gizzard troponin C-like protein with the inhibitory protein.

Electrophoresis was carried out under the same conditions as for Fig. 22. O, Origin; +, anode.

(a) 70μg of inhibitory protein

(b) 70μg of inhibitory protein + 23μg of gizzard troponin C-like protein + 5mM $CaCl_2$

(c) as (b) but with 5mM EGTA in place of $CaCl_2$

Fig. 31. Effect of gizzard troponin C-like protein on the inhibition by the inhibitory protein on the Mg^{++}-activated ATPase of reconstituted rabbit actomyosin.

ATPase activity is presented as a percentage of that obtained in the absence of the inhibitory protein, gizzard troponin C-like protein and EGTA. Assays were carried out under the standard conditions, with 384µg of reconstituted rabbit actomyosin, 91µg of gizzard tropomyosin and 53µg of inhibitory protein. ●, In the presence of 0.05mM $CaCl_2$; ○, in the presence of 1mM EGTA.

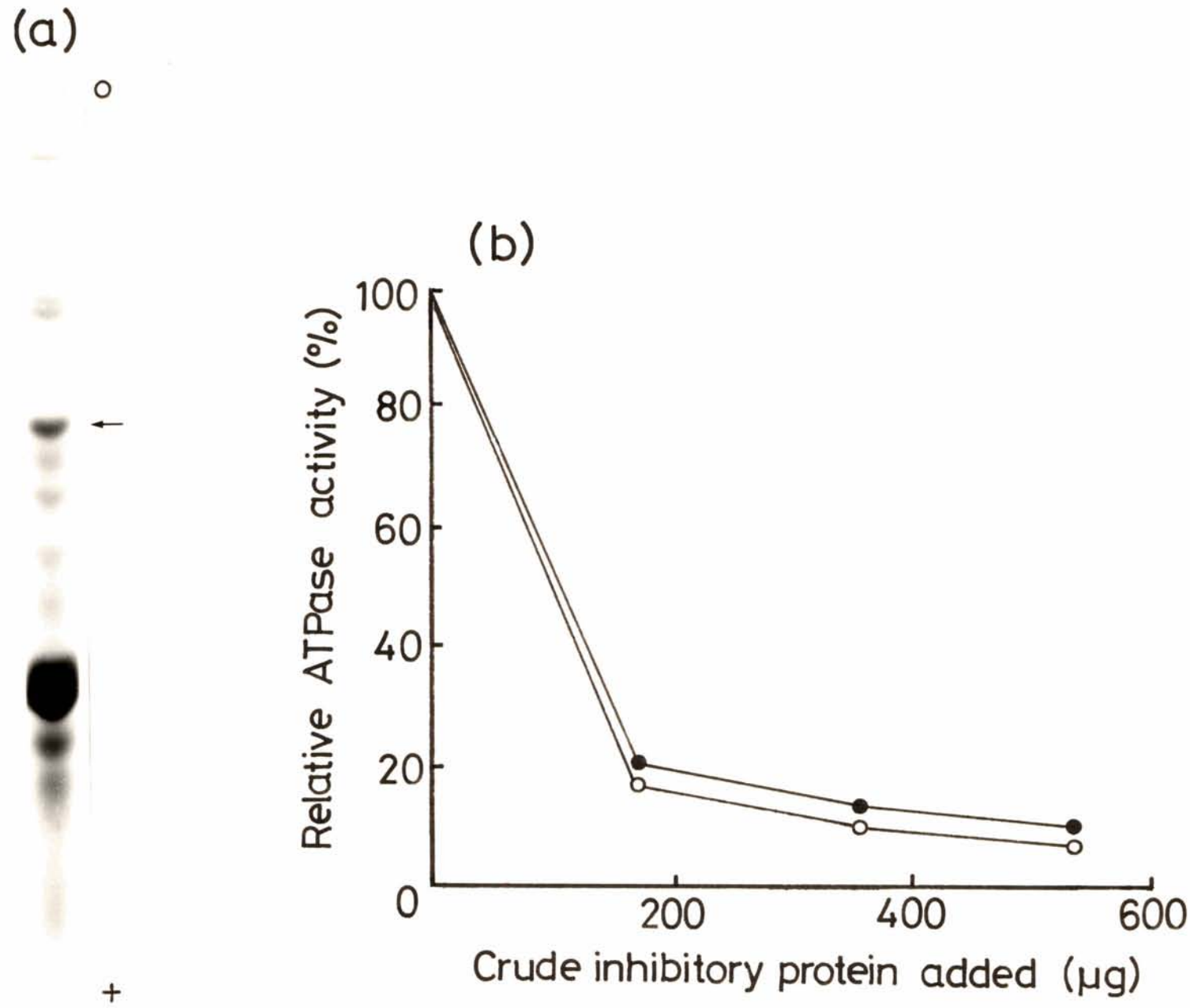

(a)
o
+
(b)
Relative ATPase activity (%)
100
80
60
40
20
0
200
400
600
Crude inhibitory protein added (μg)

Fig. 1

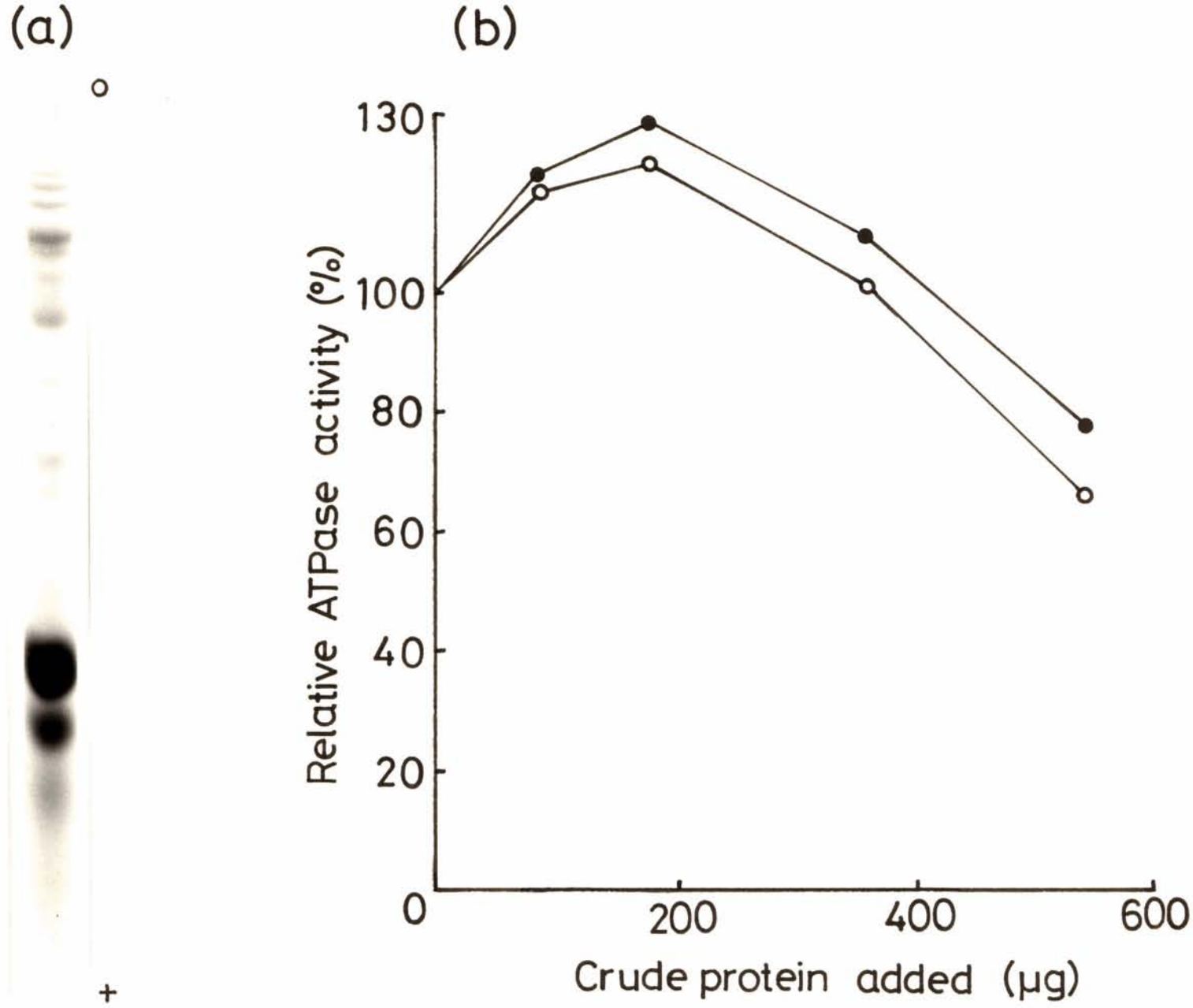

(a)
(b)
130
100
80
60
40
20
0
200
400
600
Relative ATPase activity (%)
Crude protein added (μg)

Fig. 2

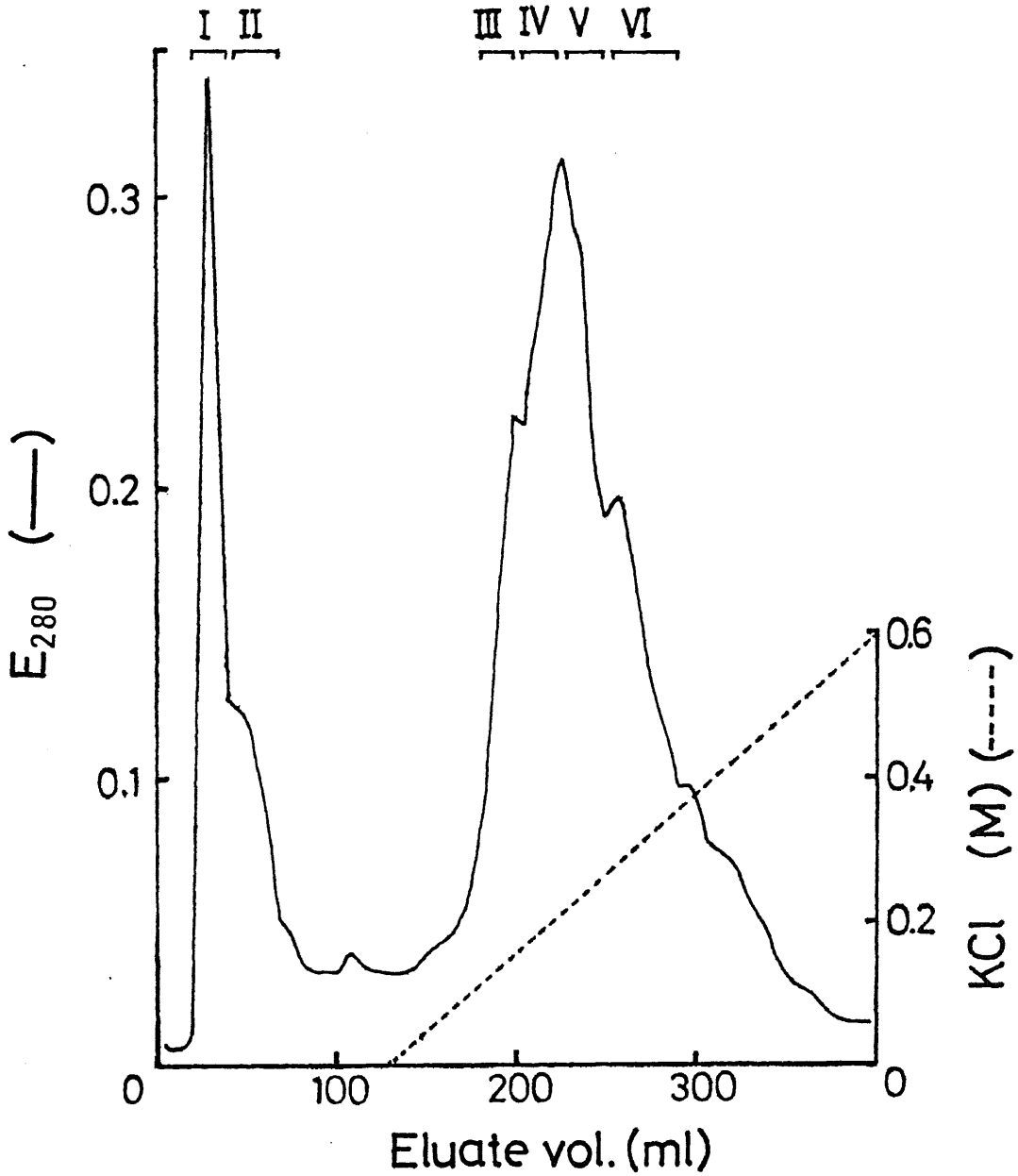

I II
III IV V VI
0.3
0.2
0.1
0
E_{280} (—)
100
200
300
0
0.6
0.4
0.2
0
KCl (M) (----)
Eluate vol. (ml)

Fig. 3

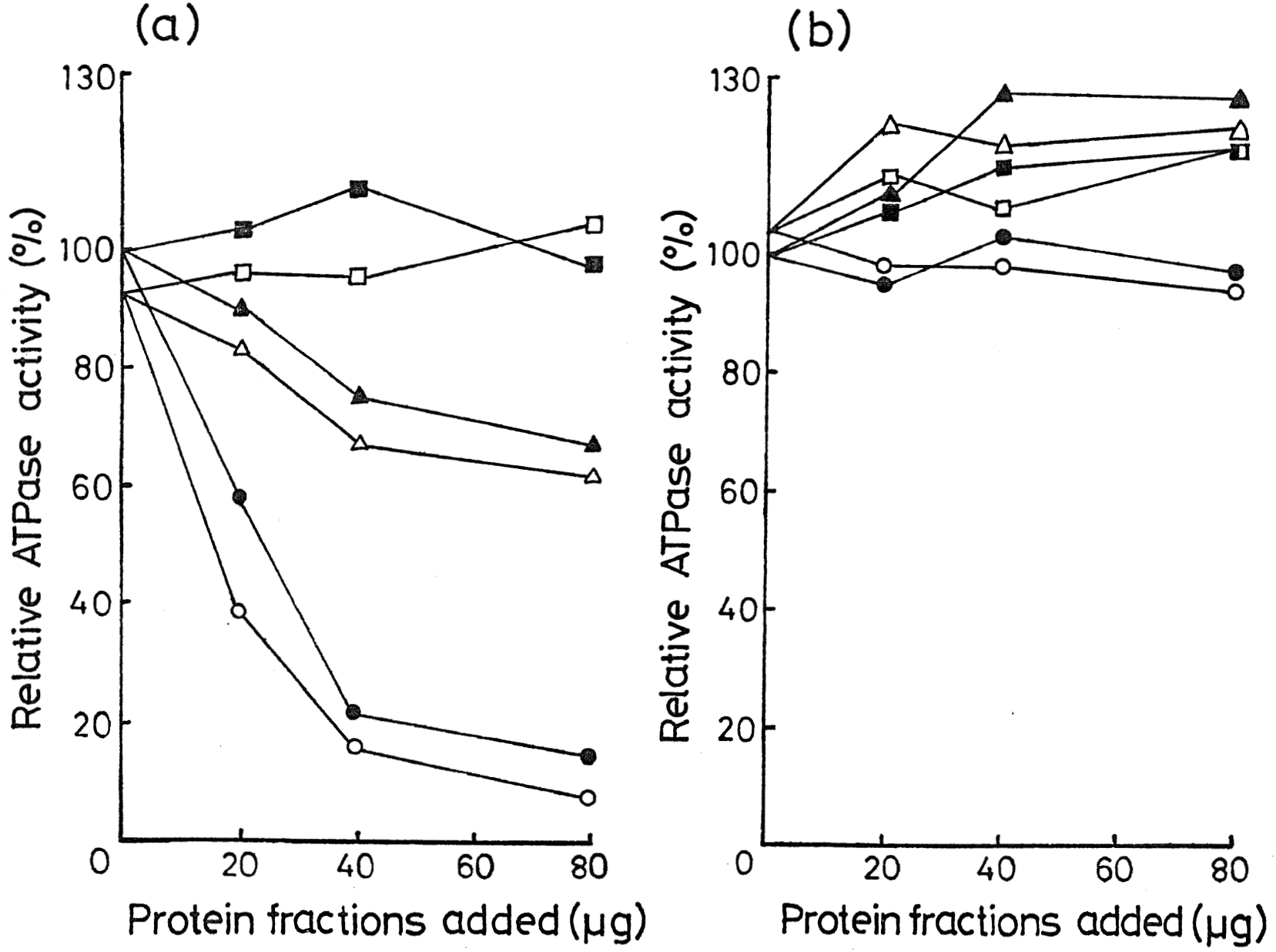

(a)
(b)
Relative ATPase activity (%)
130
100
80
60
40
20
0
20
40
60
80
Protein fractions added (μg)

Fig. 4

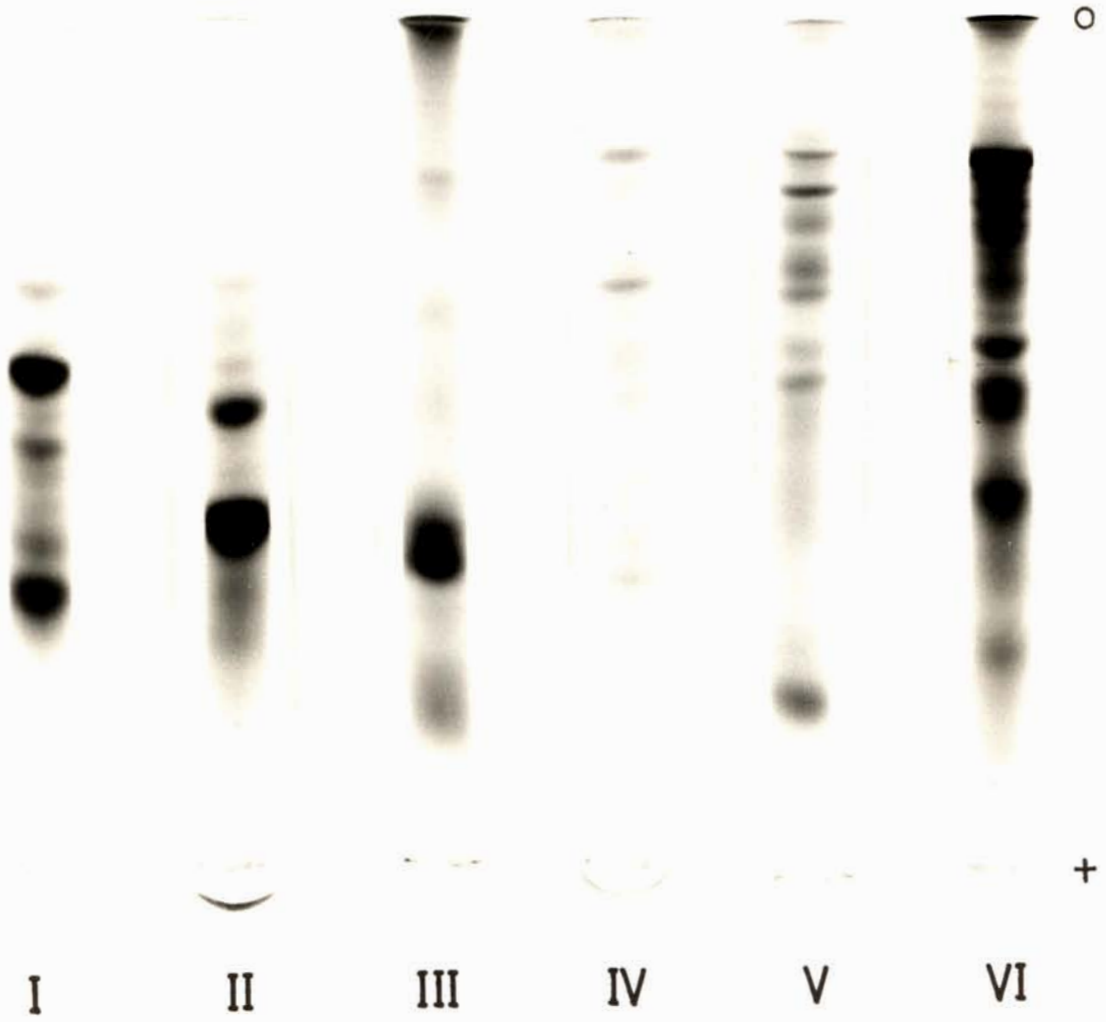


Fig. 5

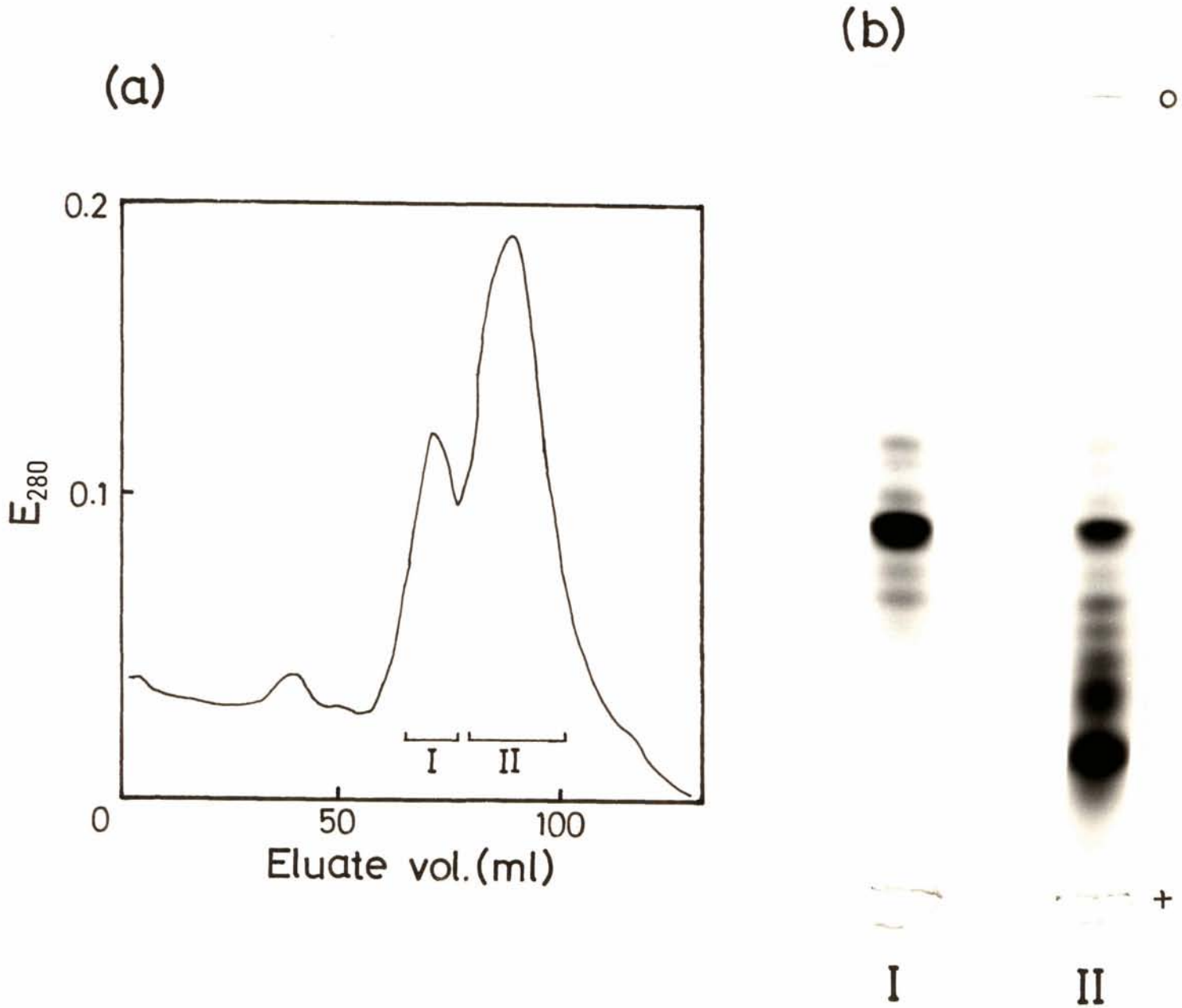


Fig. 6

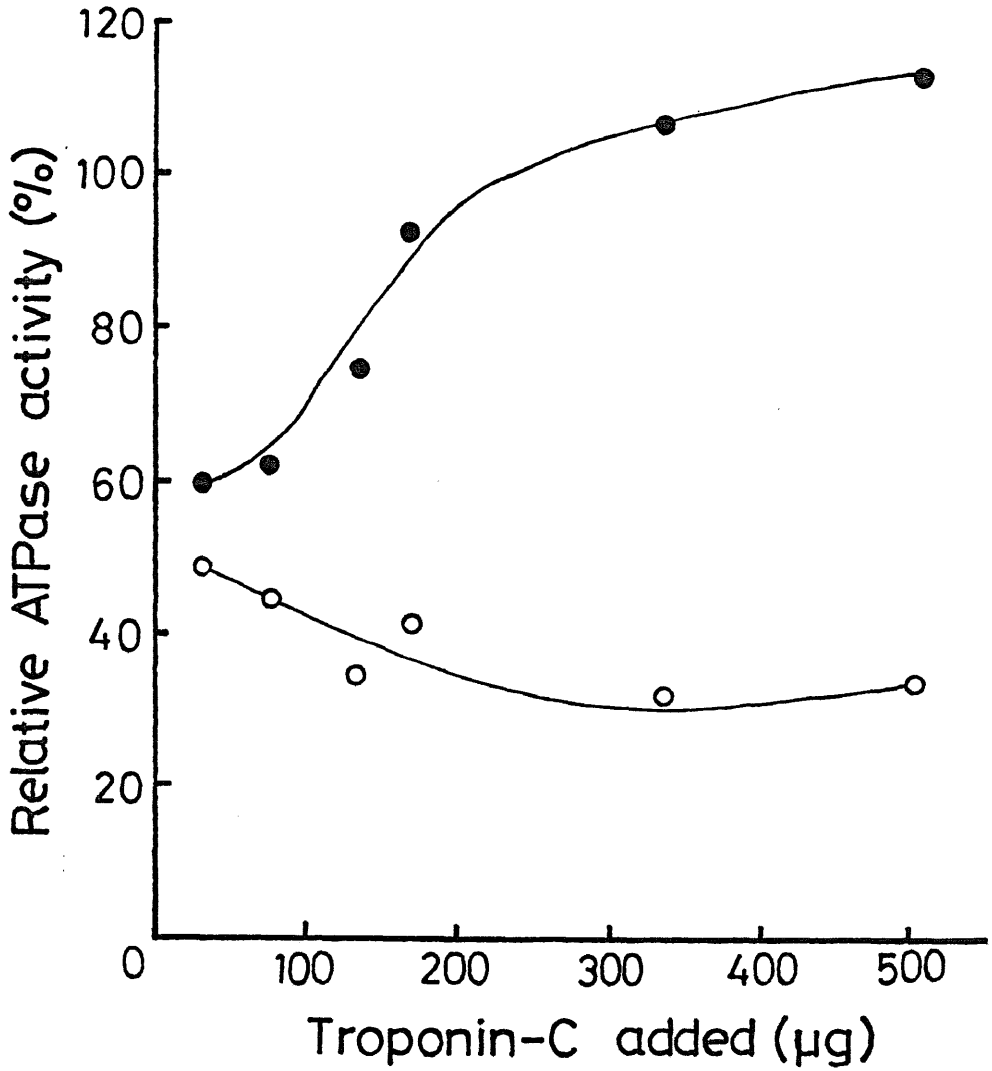

120
100
80
60
40
20
0
Relative ATPase activity (%)
100
200
300
400
500
Troponin-C added (µg)

Fig. 7

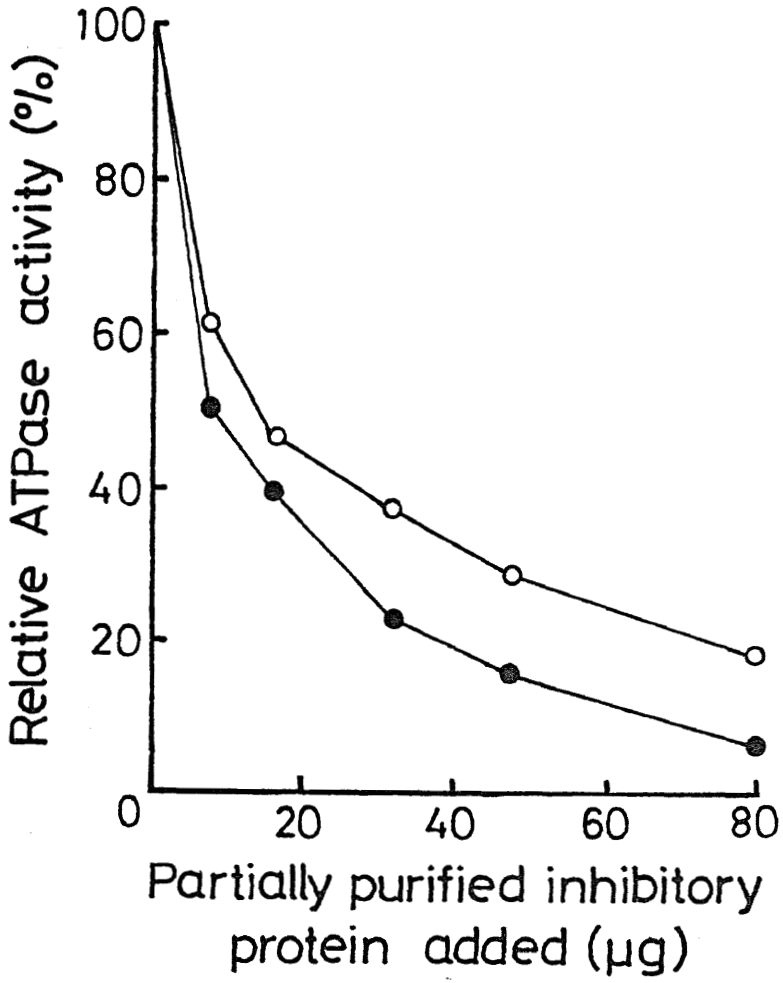

Relative ATPase activity (%)
100
80
60
40
20
0
20
40
60
80
Partially purified inhibitory protein added (μg)

Fig. 8

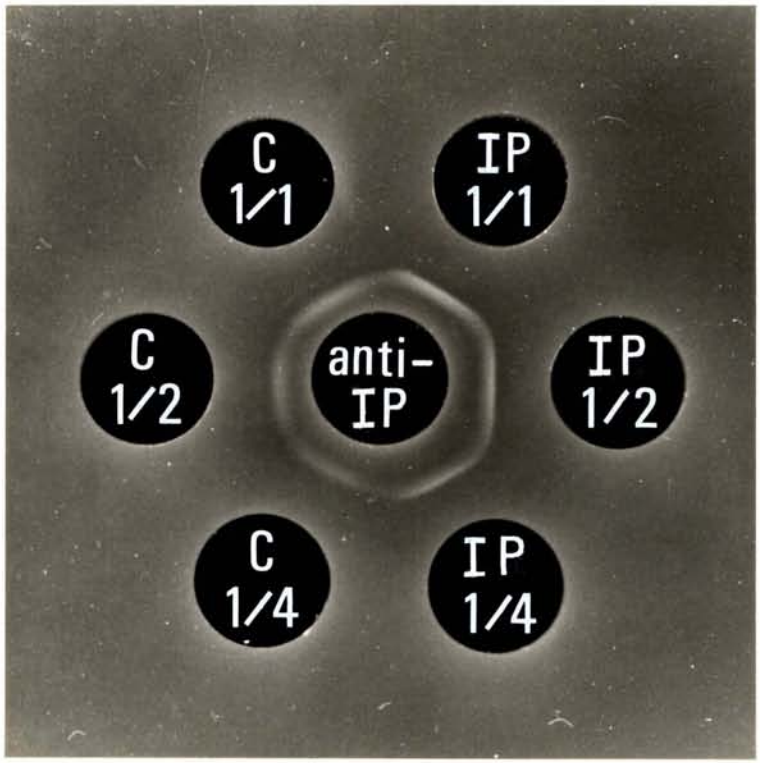
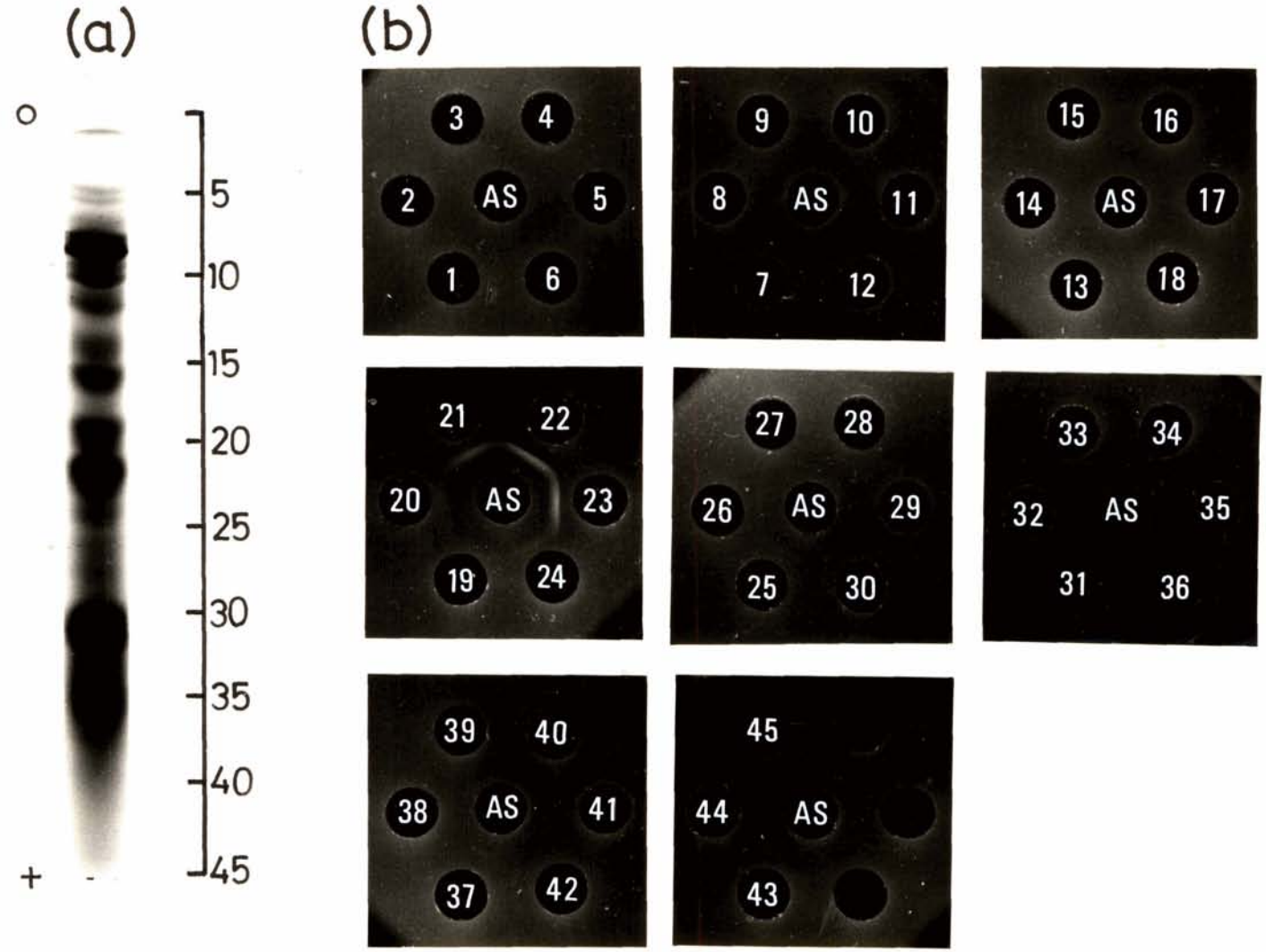


Fig. 9


(a)
(b)
5
10
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20
25
30
35
40
45
3 4 2 AS 5 1 6
9 10 8 AS 11 7 12
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21 22 20 AS 23 19 24
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Fig. 10

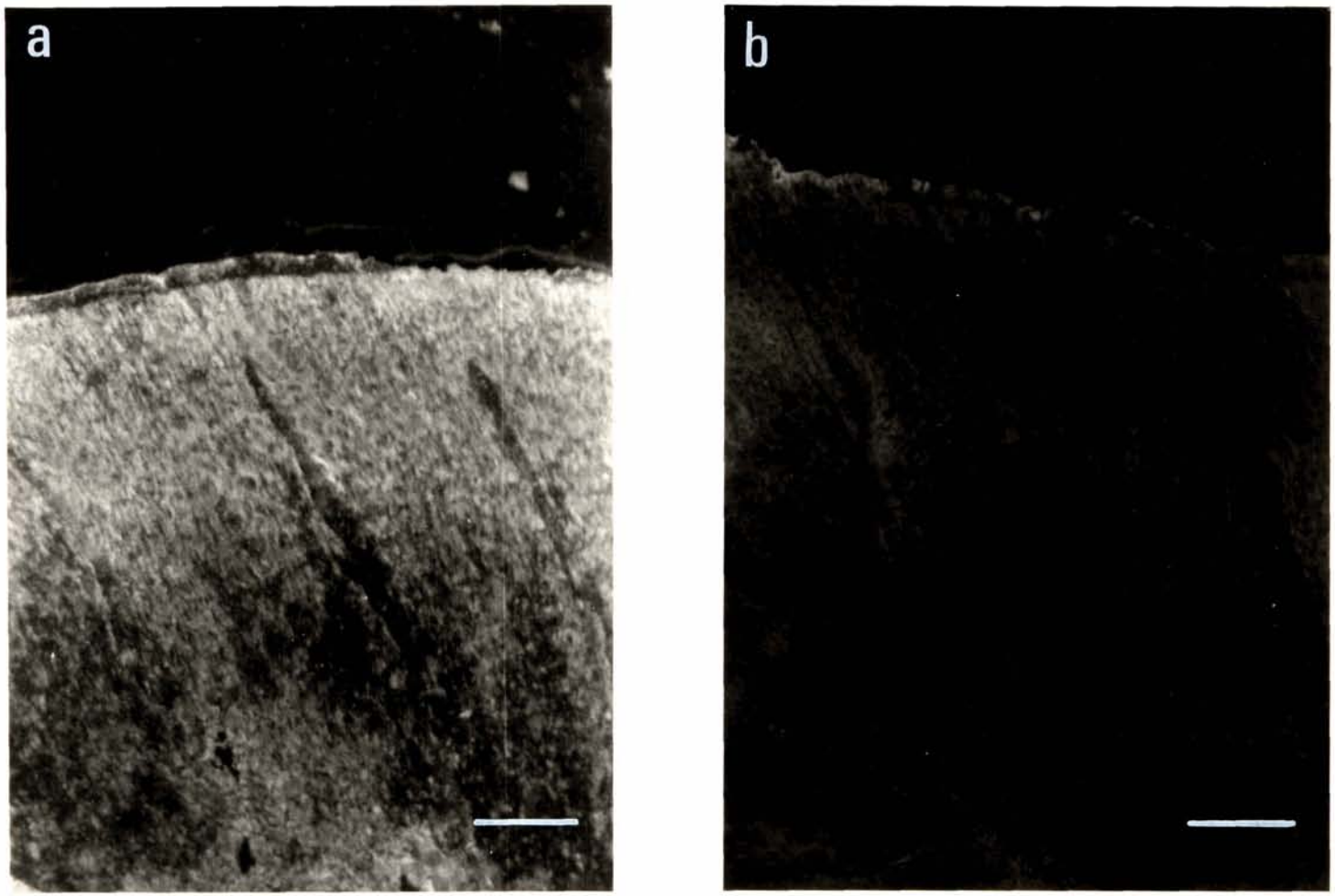


Fig. 11

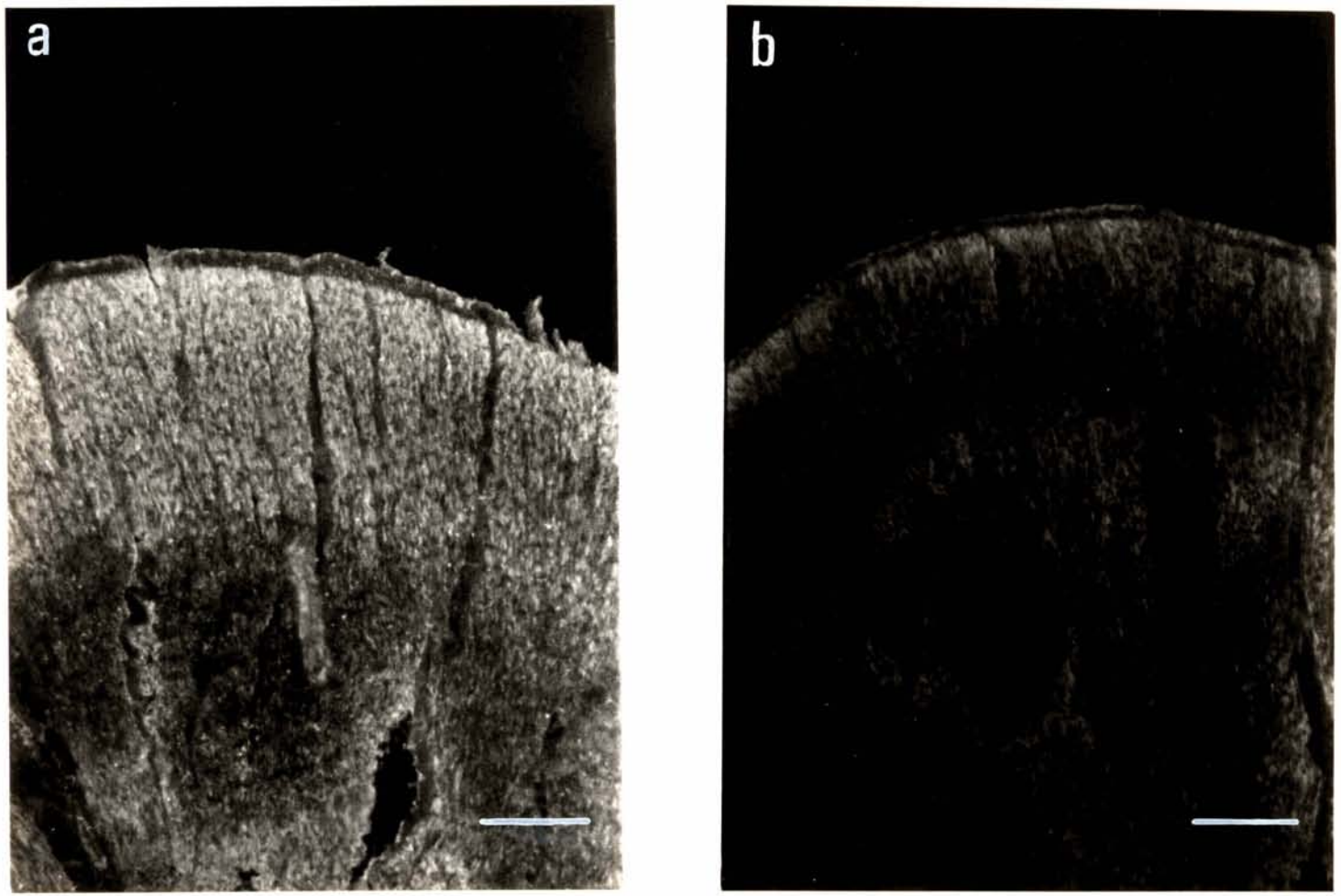


Fig. 12

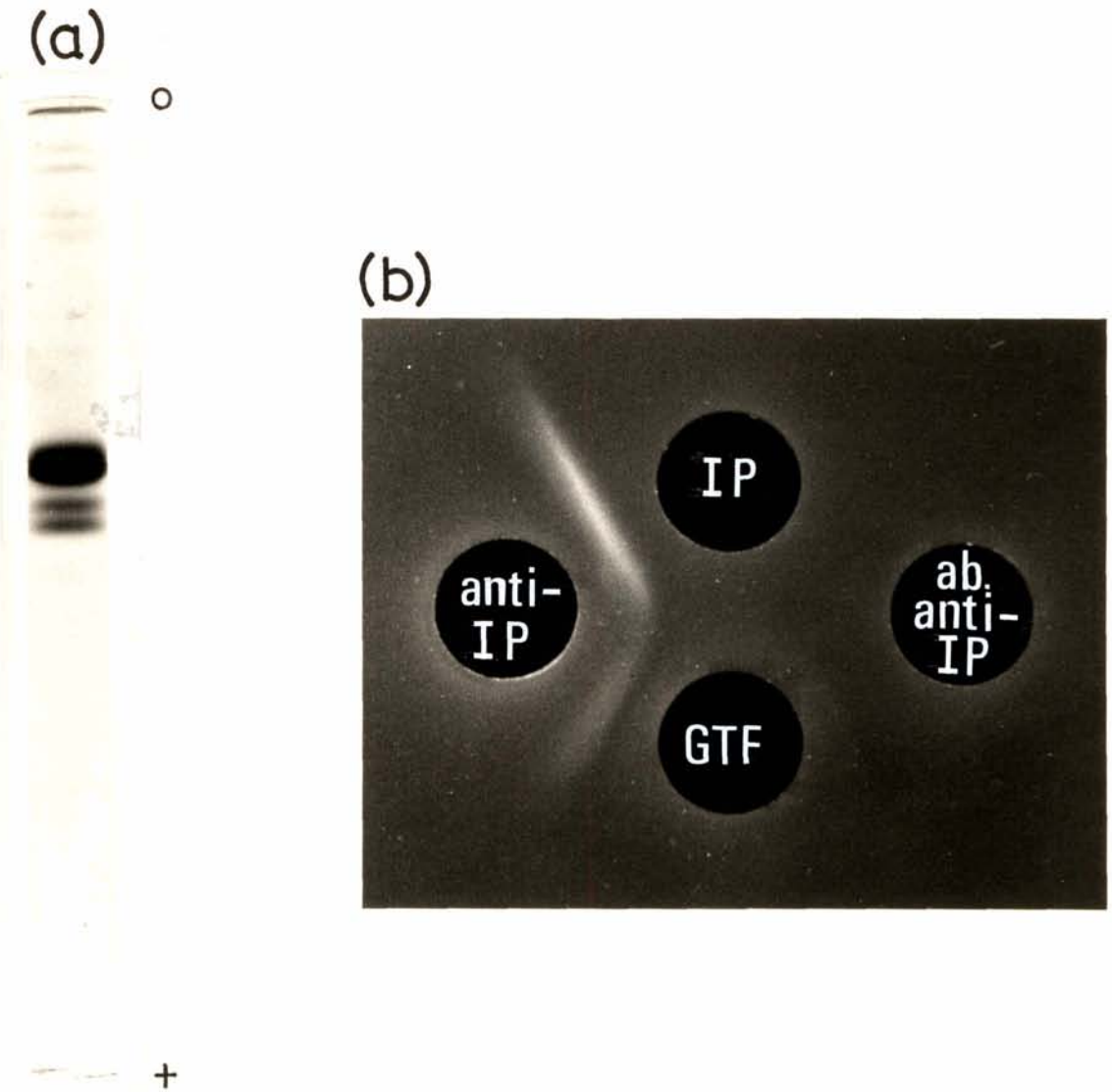


Fig. 13

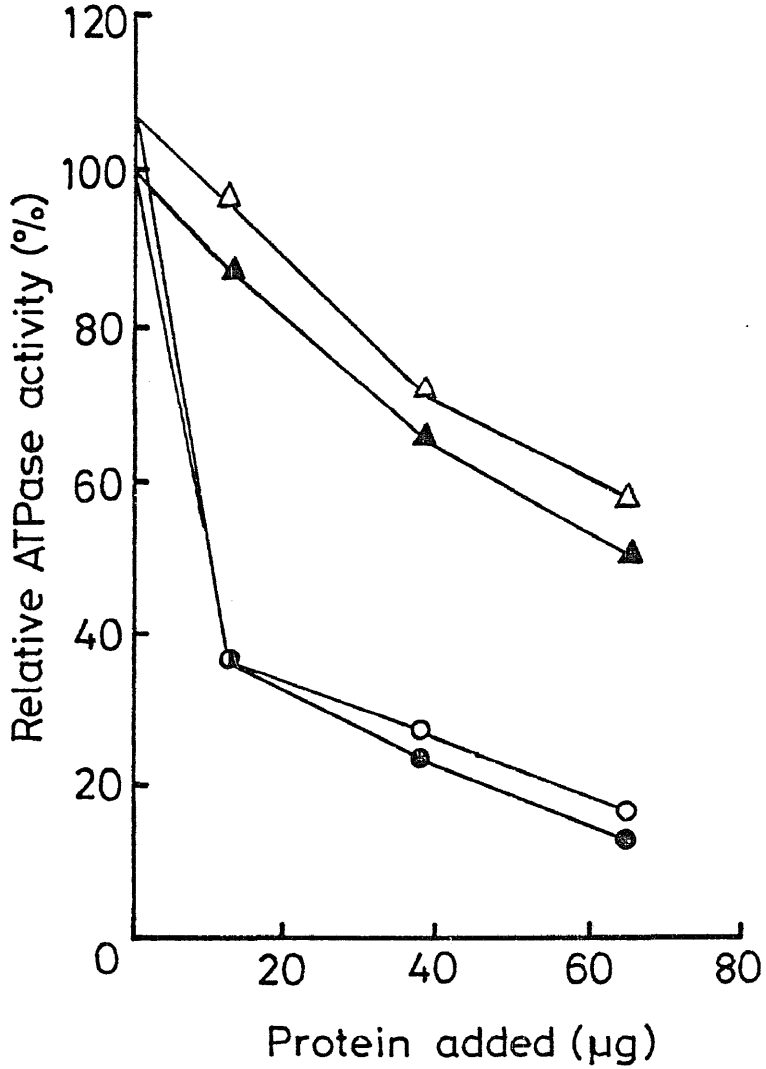

120
100
80
60
40
20
0
Relative ATPase activity (%)
20
40
60
80
Protein added (µg)

Fig. 14

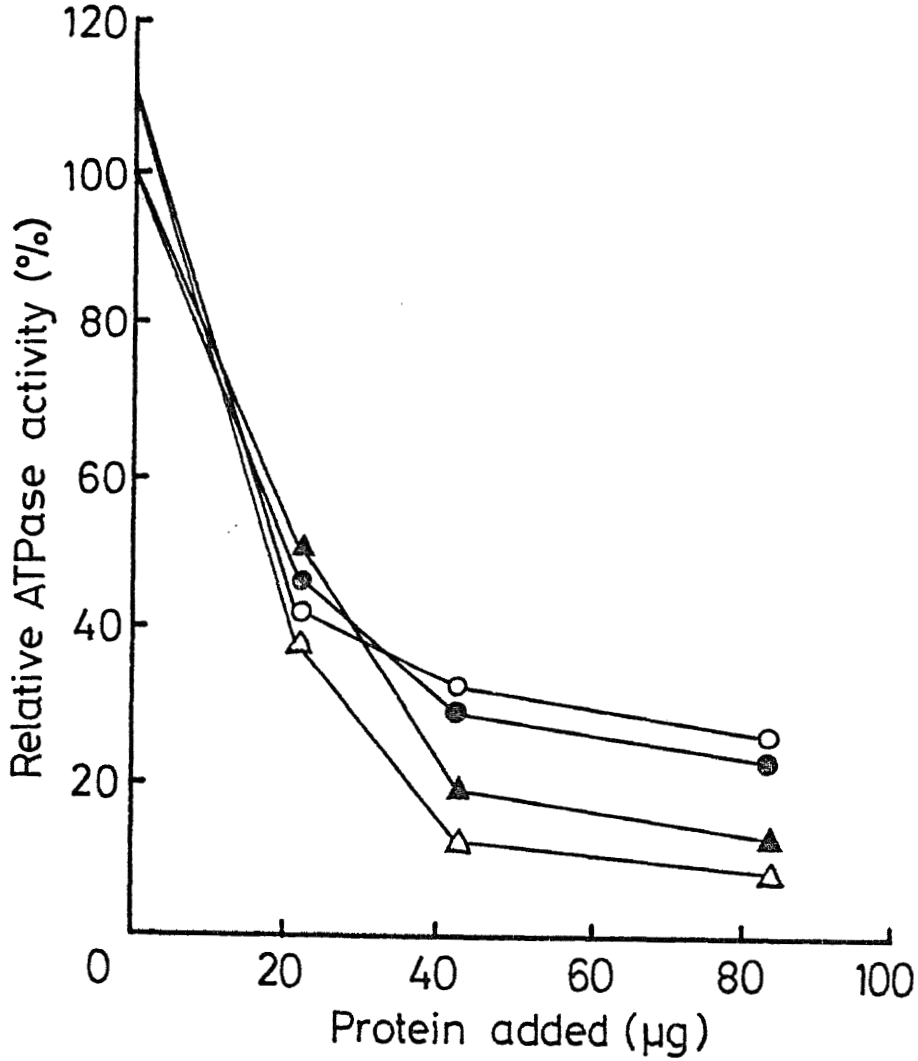

120
100
80
60
40
20
0
Relative ATPase activity (%)
20
40
60
80
100
Protein added (µg)

Fig. 15

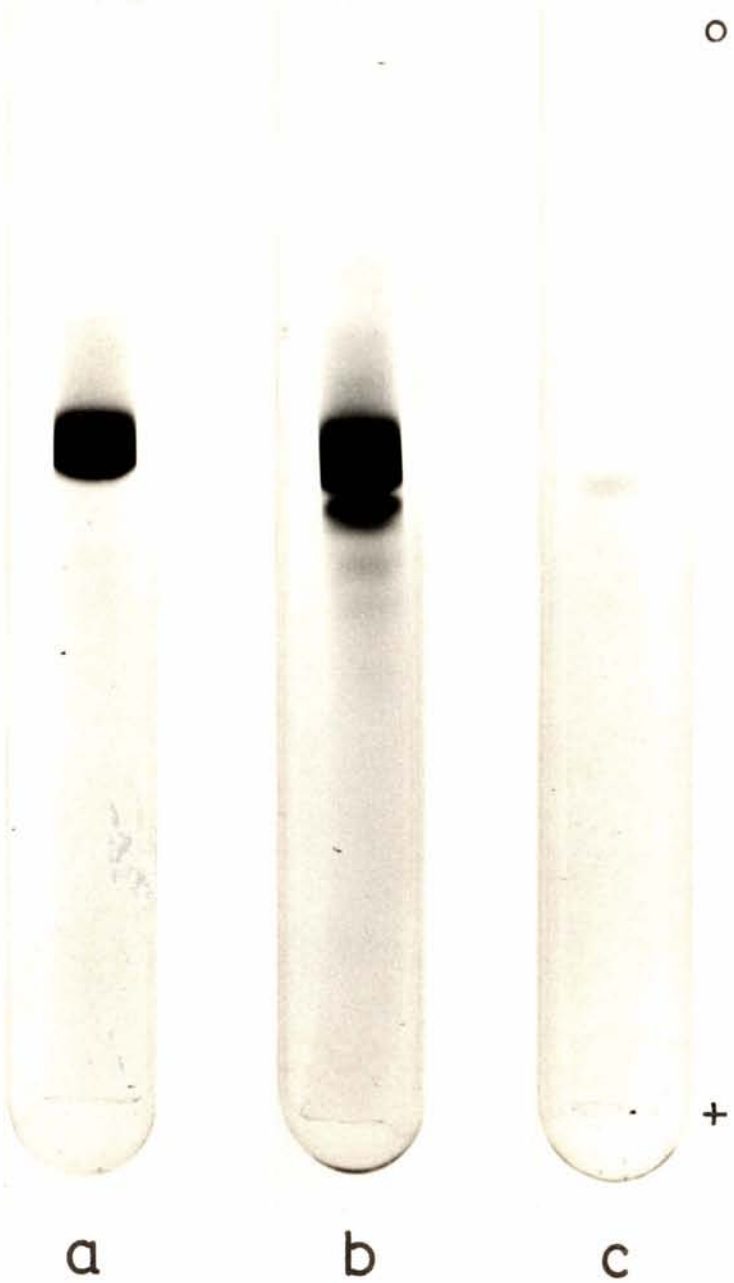


Fig. 16

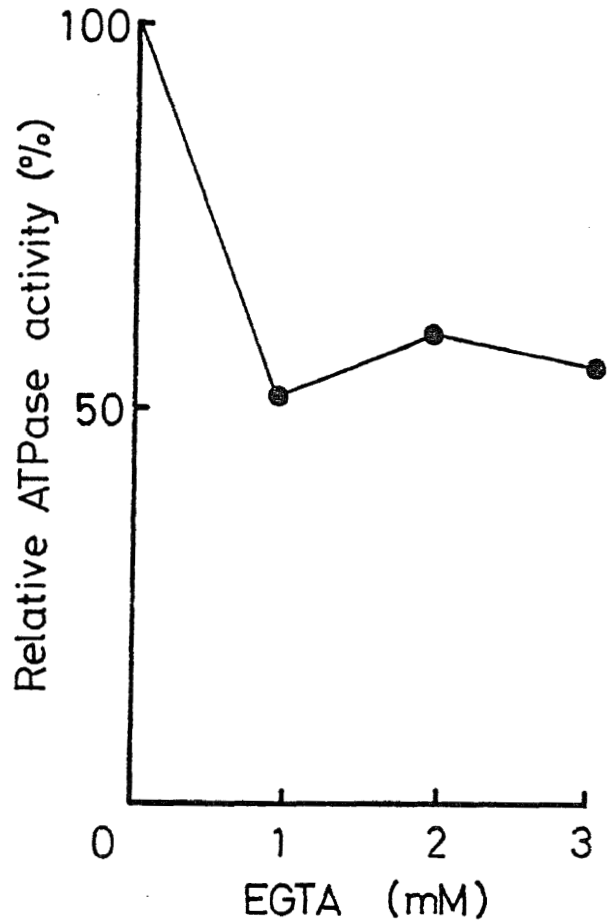


Fig. 17

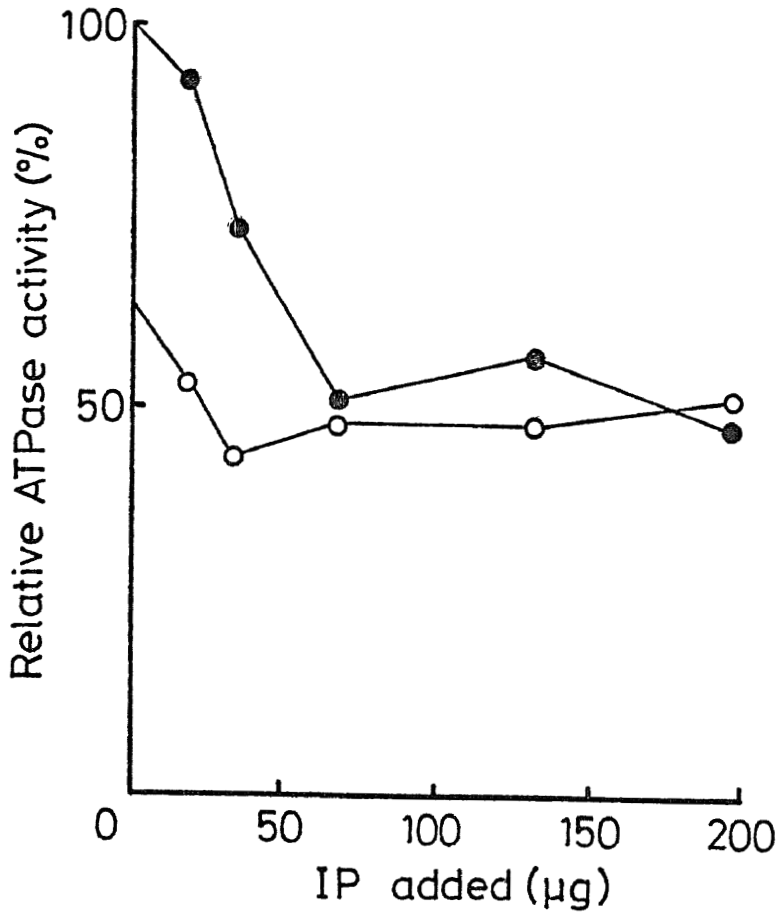
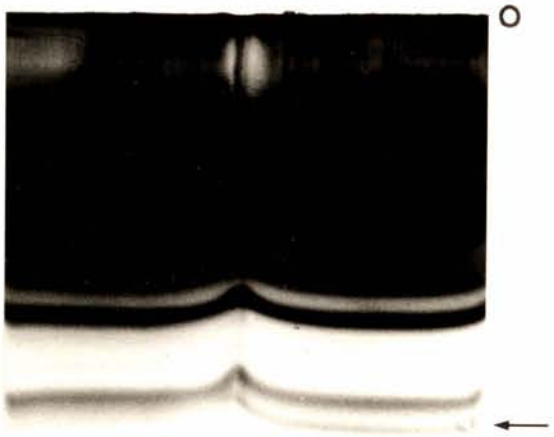


Fig. 18



a b +

Fig. 19

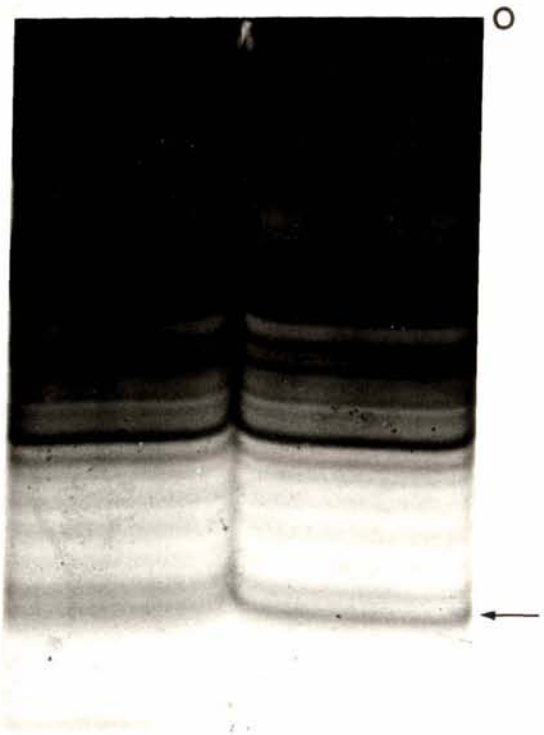


Fig. 20

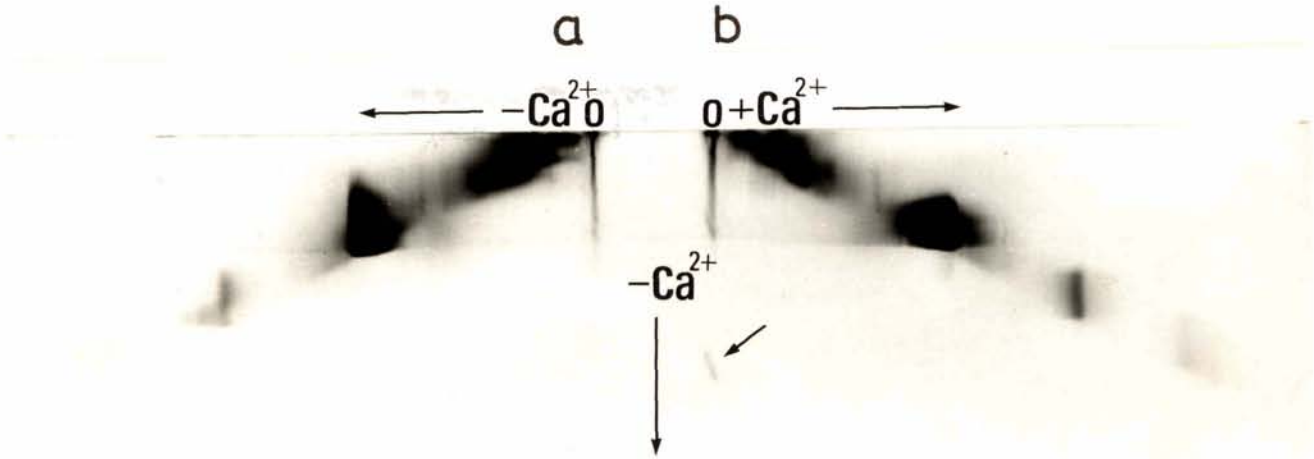


Fig. 21

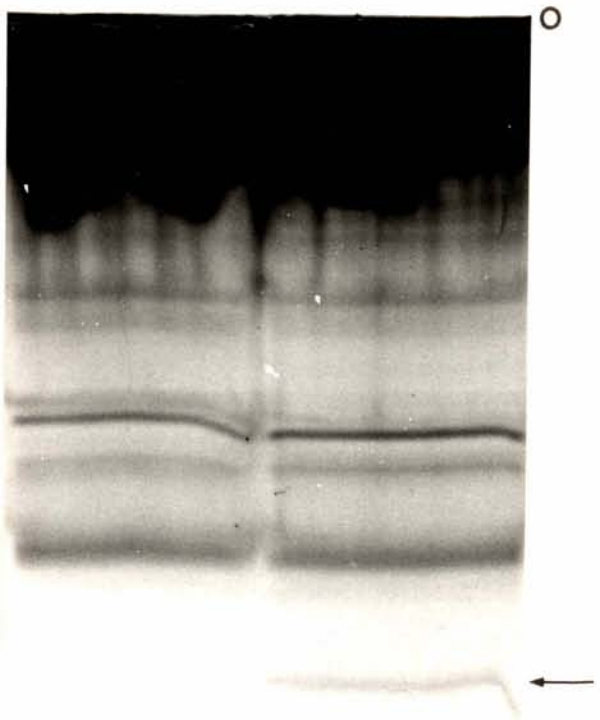


Fig. 22

(a)

o

+

(b)

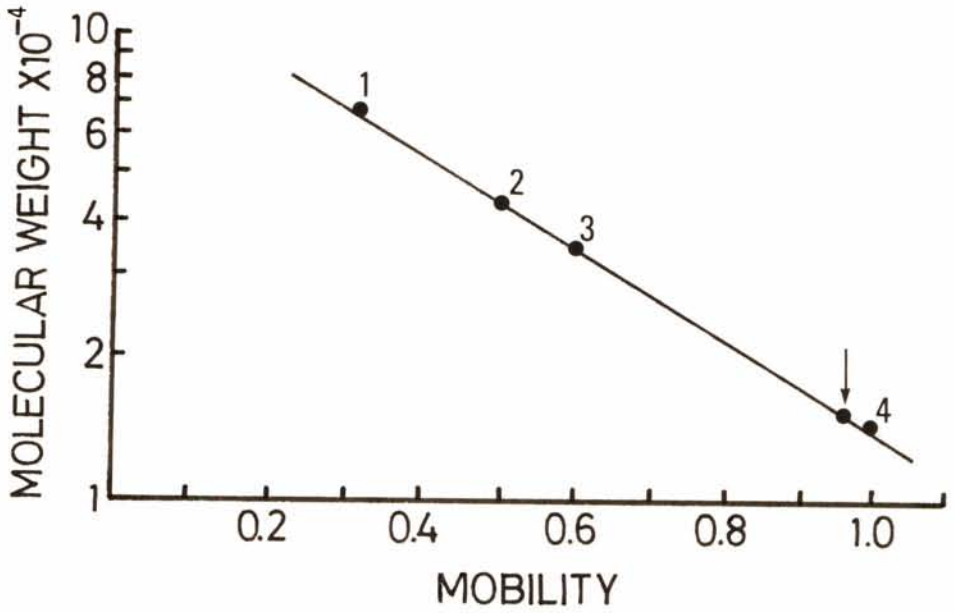


Fig. 23

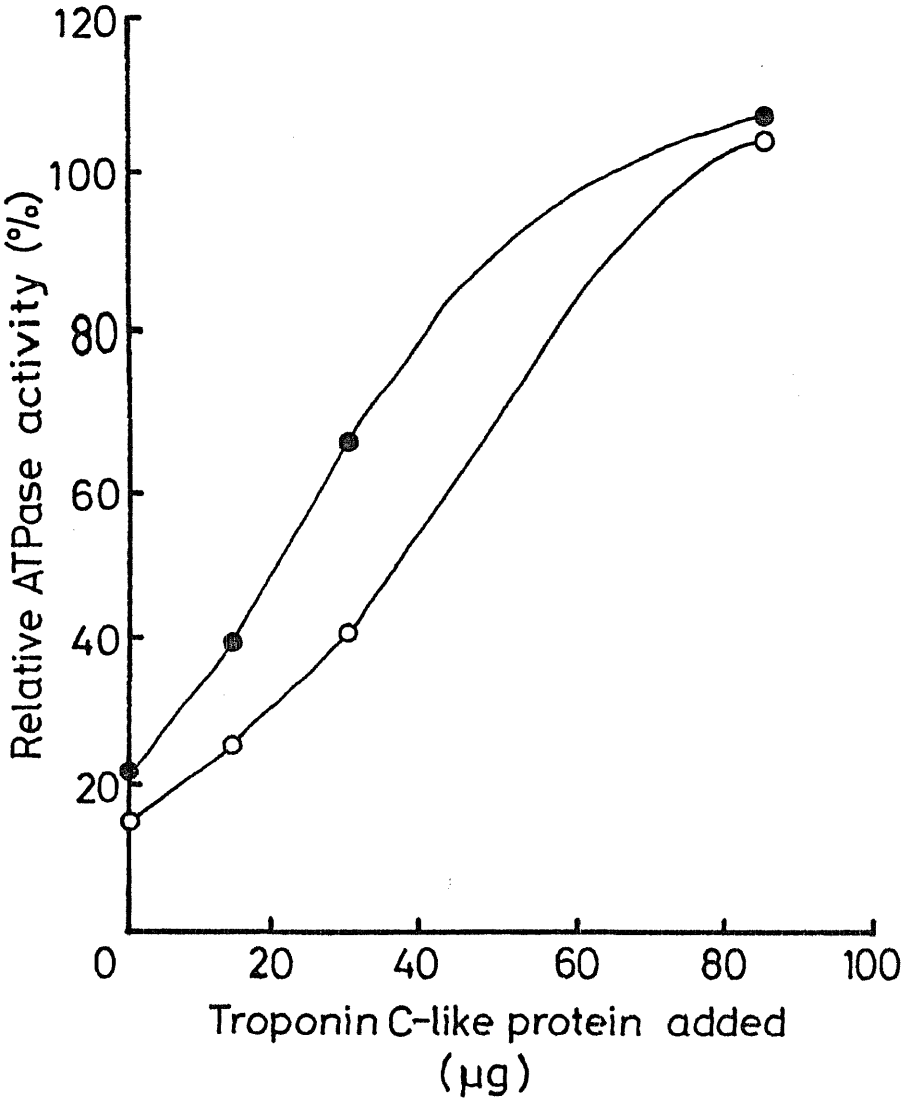

120
100
80
60
40
20
0
20
40
60
80
100
Relative ATPase activity (%)
Troponin C-like protein added
(μg)

Fig. 24

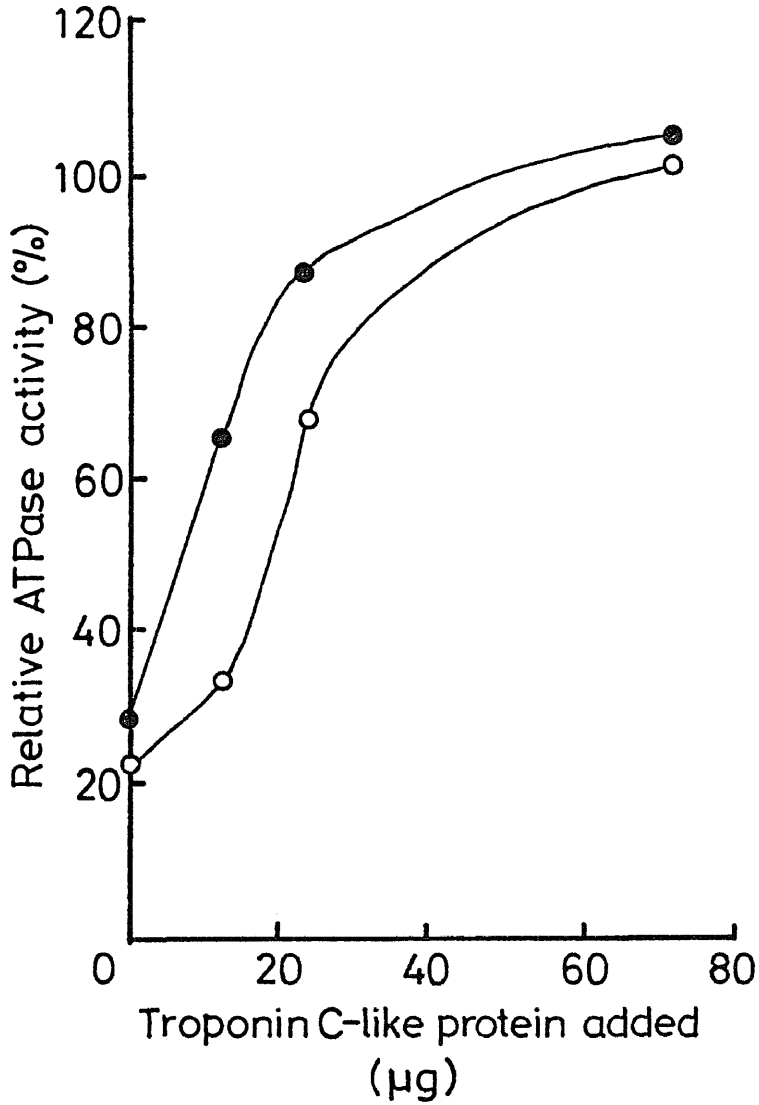

120
100
80
60
40
20
0
20
40
60
80
Relative ATPase activity (%)
Troponin C-like protein added
(μg)

Fig. 25

Fig. 26

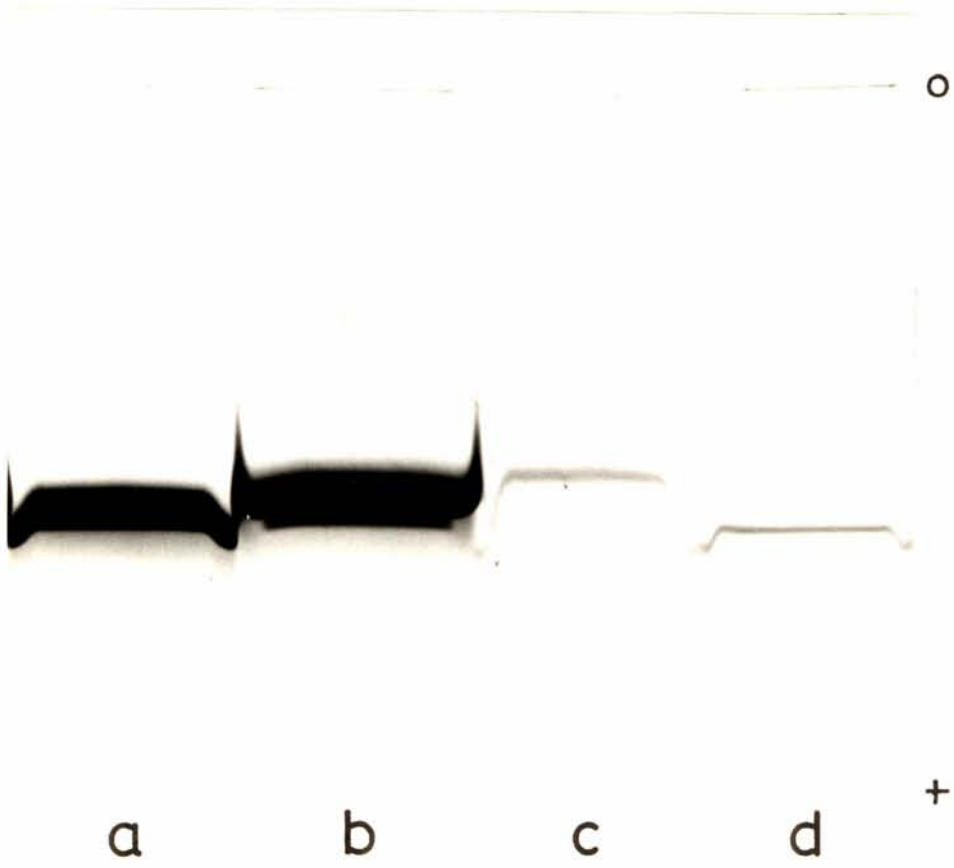


Fig. 27

Fig. 28

Fig. 29

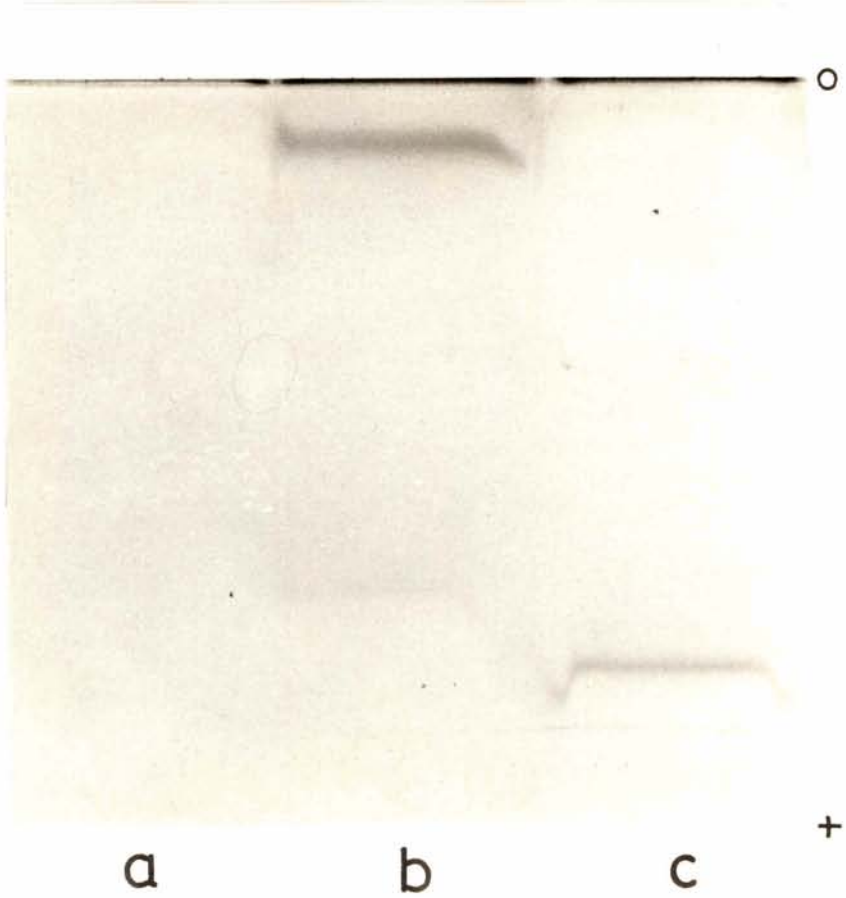


Fig. 30

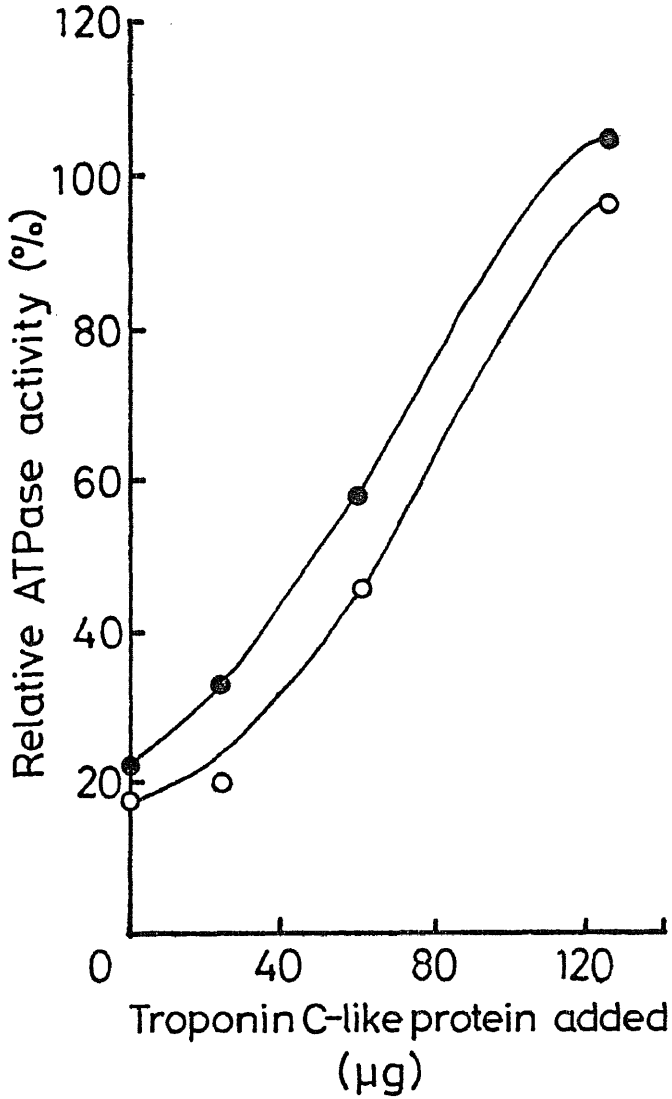

120
100
80
60
40
20
0
40
80
120
Relative ATPase activity (%)
Troponin C-like protein added
(μg)

Fig. 31

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