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Interaction of Protein-Bound Polysaccharide (PS-K) with Rabbit Skeletal Muscle Actomyosin

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The interaction of protein-bound polysaccharide (PS-K) with actomyosin from rabbit skeletal muscle was investigated by measuring the changes in adenosine triphosphatase (ATPase) activity, turbidity, and viscosity. At low ionic strengths, PS-K reduced the actin-activated Mg^{2+}-ATPase activity of myosin to 20% of the original level, whereas it slightly enhanced the myosin ATPase activity without actin. The inhibition of actin-activated ATPase activity was not reversed by increasing actin concentration and, further, the apparent affinity of myosin for actin was not altered by the addition of PS-K. The extent of inhibition depended on the sequence of addition of myosin, actin, and PS-K, *i.e.*, when PS-K was preincubated first with myosin before adding actin, the most marked inhibition was observed. The changes of actomyosin turbidity induced by PS-K were similar to those of ATPase inhibition. PS-K did not affect the polymerization and depolymerization of actin. Therefore, the inhibitory effects seem to be caused by PS-K bound to myosin filaments.

Keywords——myosin; actin; PS-K; ATPase; superprecipitation; actin polymerization

A protein-bound polysaccharide (PS-K) isolated from Basidiomycetes, has been reported to show antitumor activity against various kinds of tumor cells.[1] Although the mechanism of antitumor action of the drug is not fully understood, it is considered to be mediated by some defense mechanisms of the host, mainly consisting of immune response, because PS-K rarely shows any direct cytotoxic effect on tumor cells. Ohno *et al.*[2] reported that the addition of PS-K stimulated human lymphocytes and induced them into blastogenesis *in vitro*, as occurs with lectins. Based on studies on the interaction of lectins with cell surface receptors, various functions such as cap formation, phagocytosis, receptor movement, and mitogenesis in lymphocytes and macrophages have been shown to depend on the organization of microtubules.[3] Histochemical and electron microscopic examinations suggested the possibility of incorporation of PS-K into macrophages.[4] Recently, we found *in vitro* using porcine brain microtubule proteins that PS-K suppressed microtubule assembly in a dose-dependent manner.[5] It has been reported that thick and thin filaments also take part in various functions related to cell motility.[3,6] Many nonmuscle cells including lymphocytes and macrophages contain not only microtubule proteins but also actin and myosin. Thus, it is of great interest to determine whether PS-K interacts with actin and myosin.

In order to elucidate the possible interaction, we examined the effect of PS-K on some properties of actin, myosin, and their mixture by employing a model system using a rabbit skeletal muscle actin and myosin. Some evidence was obtained that the drug may bind to myosin filaments and inhibit both actomyosin Mg^{2+}-adenosine triphosphatase (ATPase) activity and the corresponding turbidity increase.

Materials and Methods

Preparation of Proteins——Myosin was prepared from rabbit skeletal muscle as described by Perry[7] and stored in 0.5 M KCl and 50% glycerol at −20 °C. Actin was isolated from rabbit skeletal muscle by the method of Spudich and Watt.[8] Myosin and actin were dialyzed against 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)–KOH (pH 6.5) containing 0.5 M KCl and 100 mM MES–KOH (pH 6.5), respectively, and used within 10 days. Proteins used in the present experiments were essentially pure, as determined by 8% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.[9]

Assay of ATPase Activity——ATPase activity was measured in terms of the release of inorganic phosphate.[10] Myosin ATPase activities were assayed in 0.4 ml of 50 mM or 500 mM KCl, 60 mM MES–KOH (pH 6.5), 0.2 mg/ml myosin, 2 mM adenosine triphosphate (ATP), and either 2 mM $Mg(CH_3COO)_2$, 5 mM $CaCl_2$, or 5 mM ethylenediaminetetraacetic acid (EDTA). The standard reaction mixture for actomyosin ATPase contained 60 mM MES–KOH (pH 6.5), 2 mM $Mg(CH_3COO)_2$, 2 mM ATP, 0.1 mg/ml myosin, and 0.05 mg/ml actin in a final volume of 0.4 ml. Myosin and PS-K were mixed before adding actin unless otherwise stated. Reactions were initiated by the addition of substrate and stopped after 5—30 min at 37 °C by the addition of 0.4 ml of 20% trichloroacetic acid.

Turbidity——Turbidity change was monitored at 25 °C by measuring the absorbance at 660 nm with a recording spectrophotometer (Japan Spectroscopic Co., Ltd., UVIDEC-410) equipped with a temperature-controlled cuvette chamber. The reaction mixture contained 0.15 mg of myosin and 0.09 mg of actin in 1 ml of buffer solution containing 90 mM MES–KOH (pH 6.5), 2 mM $Mg(CH_3COO)_2$, and 2 mM ATP.

Actin Polymerization——Polymerization of G-actin was followed by means of viscosity measurements at 25 °C using an Ostwald-type viscometer with a flow time for buffer of 48 s. The solution contained 0.45 mg/ml actin, 50 mM KCl, 20 mM MES–KOH (pH 6.5), 1 mM $Mg(CH_3COO)_2$, and 0.45 mM ATP. The viscosity was expressed as specific viscosity (η_{sp}).

Protein Determination——Protein was determined by the method of Lowry *et al.*[11] with bovine serum albumin as a standard.

Results

Effect of PS-K on Myosin ATPase

We investigated the effect of PS-K on myosin ATPase activities (Table I). The Mg^{2+}- and Ca^{2+}-ATPase activity of myosin were both slightly enhanced by adding PS-K in the presence of KCl at both concentrations of 50 and 500 mM. On the other hand, the drug appeared to inhibit EDTA-ATPase activity at 50 mM KCl, while it did not affect the activity at 500 mM KCl. Since PS-K *per se* did not show any ATPase activity, this result may reflect the interaction between the drug and myosin.

Effect of PS-K on Actomyosin ATPase

At low ionic strengths, F-actin is capable of stimulating myosin Mg^{2+}-ATPase activity.

TABLE I. Myosin ATPase Activity in the Presence or Absence of PS-K

Added KCl (mM)	PS-K (mg/ml)	ATPase activity (nmol/mg·min)		
		2 mM $Mg(CH_3COO)_2$	5 mM $CaCl_2$	5 mM EDTA
500	0	15.4	323	1195
	0.4	16.7	372	1262
	0.8	17.2	398	1170
50	0	33.8	825	37.6
	0.4	46.2	893	31.1
	0.8	50.0	952	27.3

ATPase activity was measured at 37 °C in 0.4 ml of 50 mM or 500 mM KCl, 60 mM MES–KOH (pH 6.5), and 2 mM ATP; in addition, 2 mM $Mg(CH_3COO)_2$, 5 mM $CaCl_2$, and 5 mM EDTA (final concentrations) were added to the reaction medium for Mg^{2+}-ATPase, Ca^{2+}-ATPase, and EDTA-ATPase, respectively. The concentration of myosin was 0.2 mg/ml.

The enhancement of the activity by actin was about 12-fold under the present conditions. The addition of PS-K strongly inhibited the actin-activated Mg^{2+}-ATPase activity of myosin (Fig. 1). The extent of inhibition increased with increasing amount of PS-K added and at maximum about 80% of the original activity in the absence of the drug was inhibited. The amounts of PS-K required for half-maximal and maximal inhibition were 0.03 and 0.2 mg/ml, respectively, in the presence of 0.1 mg/ml myosin and 0.05 mg/ml actin. These values were lower than those obtained when PS-K enhanced myosin Mg^{2+}-ATPase activity, *i.e.*, the concentrations of PS-K which showed half-maximal and maximal activation were about 0.2 and 0.6 mg/ml, respectively.

Some Properties of PS-K Inhibition of Actomyosin ATPase

Myosin Mg^{2+}-ATPase activity in the presence and absence of PS-K is illustrated as a function of actin concentration in Fig. 2a. The inhibitory effect of PS-K was observed

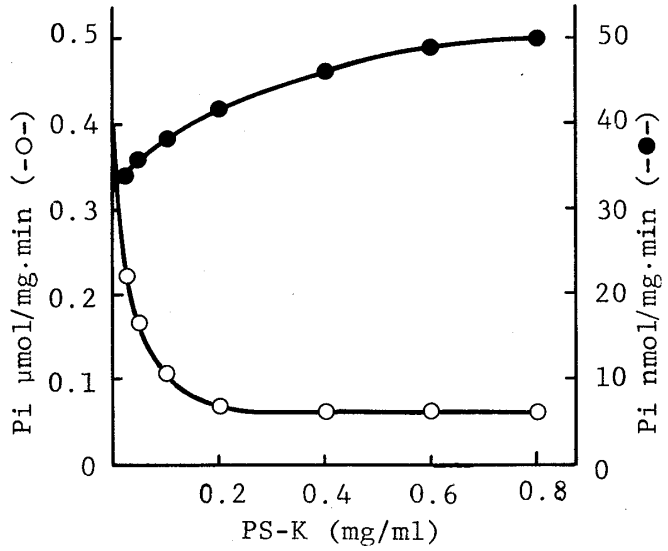

Fig. 1. Effect of PS-K on Myosin Mg^{2+}-ATPase Activity in the Presence or Absence of Actin

ATPase activity was measured as described in Materials and Methods, in media containing 60 mM MES-KOH (pH 6.5), 2 mM $Mg(CH_3COO)_2$, and 2 mM ATP, made up to contain the concentration of PS-K indicated. The concentrations of myosin and actin were 0.1 and 0.05 mg/ml, respectively. The incubation times were 5 min for actomyosin ATPase (○) and 30 min for myosin ATPase assay (●).

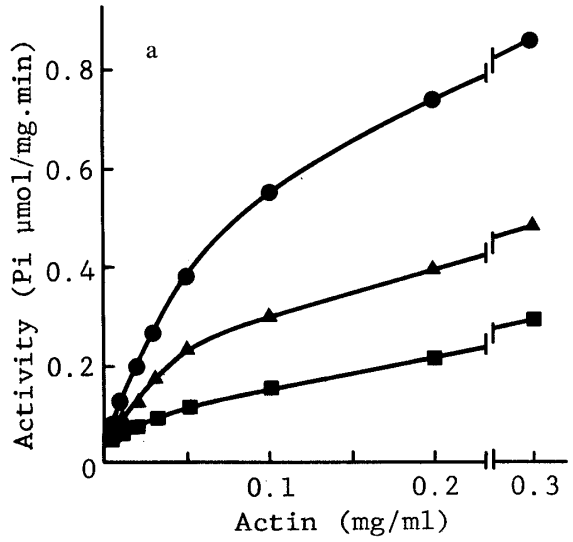

Fig. 2a. Dependence on Actin Concentration of Myosin ATPase Activity in the Presence or Absence of PS-K

Enzyme activity was measured under the standard conditions modified to contain the indicated concentration of actin. ●, none; ▲, 0.025; ■, 0.1 mg/ml PS-K.

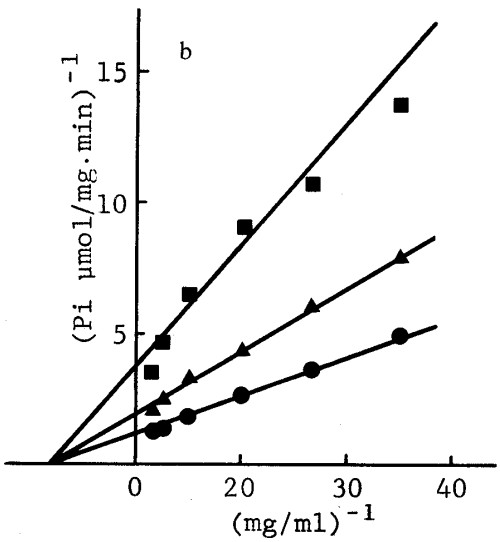

Fig. 2b. Double Reciprocal Plots of the Data in Fig. 2a.

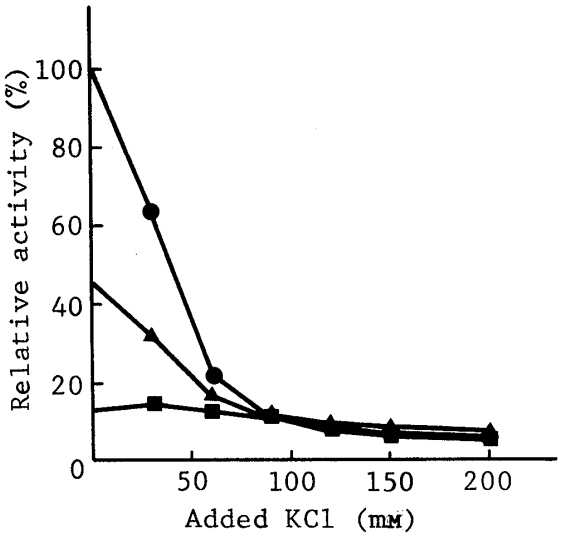

Fig. 3. Effect of KCl on Actomyosin Mg^{2+}-ATPase Inhibition by PS-K

The reaction conditions were the same as for Fig. 1. The concentration of KCl was varied as shown. ●, none; ▲, 0.05; ■, 0.2 mg/ml PS-K.

TABLE II. Effect of Sequence of Addition on the ATPase Activity of Mixtures of Myosin, Actin, and PS-K

Conditions	Inhibition (%)		
	PS-K (mg/ml)		
	0.05	0.1	0.2
(Myosin + PS-K) + actin	57.7	73.1	82.7
(PS-K + actin) + myosin	48.4	64.1	70.7
(Myosin + actin) + PS-K	31.7	40.7	54.0

Experimental conditions were the same as for Fig. 1. Parentheses indicate that the materials enclosed were preincubated together for 3 min at 37 °C before addition of the remainder.

whenever actin was presented in the assay mixture. Addition of excess actin (more than 10 times by weight with respect to PS-K) was not able to overcome the inhibition. Double-reciprocal plots showed that the presence of PS-K barely changed the apparent affinity of myosin for actin (Fig. 2b).

Figure 3 shows the effect of KCl on the inhibition of actomyosin Mg^{2+}-ATPase by PS-K. Both the specific activity and the extent of inhibition by PS-K decreased with increasing concentration of KCl added. The inhibitory effect was not observed when more than 90 mM KCl was added to the standard reaction mixture, indicating that the drug is not able under these conditions to suppress actin activation of myosin ATPase activity. Similar phenomena were observed when NaCl was used instead of KCl. In addition, the inhibition by PS-K was observed over a wide pH range (pH 6—8.5), and was independent of temperature (10—37 °C).

Table II illustrates that the extent of inhibition by PS-K varied depending on the sequence of addition of reaction components. Incubation of PS-K with myosin for 3 min at 37 °C prior to the addition of actin resulted in the greatest inhibition, presumably because myosin was in the form of myosin–PS-K complex and not available for acting binding. Fifty per cent inhibition was observed when PS-K (0.2 mg/ml) was added last, probably because myosin was already in the form of myosin–actin complex. Even so, it appeared that PS-K could still form some kind of complex with myosin.

Effect of PS-K on Turbidity Change

Superprecipitation, turbidity increase of actomyosin by Mg^{2+}-ATP, is closely related to

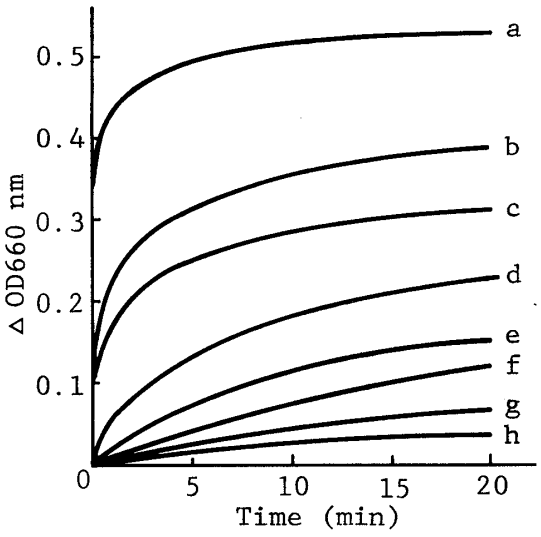

Fig. 4. Effect of PS-K on the Turbidity Change of Actomyosin Induced by Mg^{2+}-ATP

The sequence of addition of proteins was the same as in the assay of ATPase activity and the incubation conditions were those given in Materials and Methods. a, none; b, 0.005; c, 0.01; d, 0.02; e, 0.03; f, 0.04; g, 0.06; h, 0.08 mg/ml PS-K.

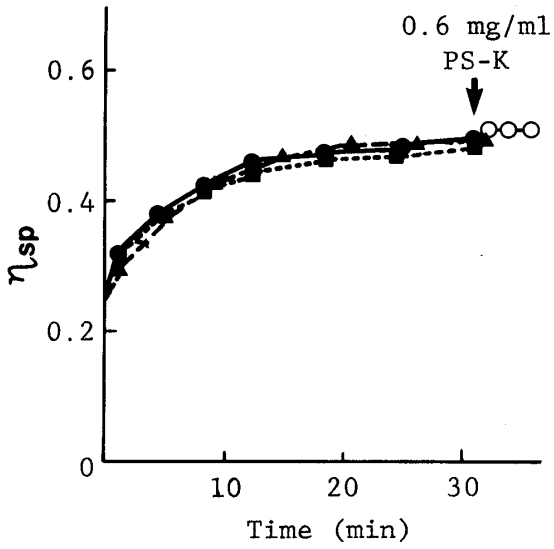

Fig. 5. Effect of PS-K on Actin Polymerization

Experimental conditions were as described in Materials and Methods. ●—●, none; ▲---▲, 0.3; ■---■, 0.6 mg/ml PS-K.

the actin-activated Mg^{2+}-ATPase activity of myosin. When myosin was mixed with PS-K at low ionic strengths, ATP did not have any significant effect on the turbidity, as in the case of myosin–actin mixture. The addition of PS-K suppressed both the initial velocity and the extent of turbidity increase in a dose-dependent fashion (Fig. 4). Furthermore, the extent of inhibition was altered by the sequence of addition of reaction components in the same way as with ATPase inhibition.

Effect of PS-K on Actin Polymerization

We examined the effect of PS-K on the conversion of G-actin to F-actin by viscometry. As shown in Fig. 5, PS-K, which has slight viscosity in itself, has no inhibitory effect on the polymerization of G-actin under conditions such that its concentration is sufficient to suppress actomyosin ATPase activity and turbidity increase. PS-K did not show any change of viscosity during incubation with ATP. In addition, the drug did not induce depolymerization of F-actin.

Discussion

The present results provide convincing evidence for the interaction of PS-K with skeletal muscle actomyosin. The striking features of PS-K are its remarkable inhibition of the actin-activated Mg^{2+}-ATPase activity of myosin and of the corresponding turbidity increase. These inhibitory effects are thought to occur through the binding of the drug to myosin filaments. In fact, the existence of such an interaction is suggested by the following findings. 1) Addition of PS-K led to a slight enhancement of myosin Mg^{2+}- or Ca^{2+}-ATPase activity in the absence of actin (Table I). Moreover, the interaction seems to be tight, because the activation was still observable even at 500 mM KCl. 2) If PS-K acted directly on the actin molecule, large quantities of actin would eliminate the ATPase inhibition by PS-K. However, considerable inhibition was observed even when the ratio of actin to PS-K was over 10 (w/w) (Fig. 2). In addition, the drug had little effect on the polymerization and depolymerization of actin (Fig.

5). 3) The order of addition of myosin, actin, and PS-K affected the PS-K inhibition of ATPase activity and turbidity change (Table II). When PS-K was preincubated with myosin before the addition of actin, the inhibitory action was the greatest, whereas other combinations resulted in partial inhibition.

Brain tubulin, a main component of microtubule proteins, has been reported to inhibit both actomyosin Mg^{2+}-ATPase activity and superprecipitation through binding to myosin filaments.[9,12] The interaction is very similar to the myosin–PS-K interaction in some respects, such as 2) and 3), though tubulin, which is distinct from PS-K, was less effective as an inhibitor of actomyosin ATPase activity and turbidity change, and had no effect on myosin ATPase activity. It has been demonstrated that microtubule proteins contain, in addition to tubulin, a heterogeneous population of accesory proteins called microtubule-associated proteins (MAPs) which are capable of stimulating microtubule assembly *in vitro*.[13] Recently, we proposed a mechanism for the interaction of PS-K with microtubule proteins, *i.e.*, the drug interacts with MAPs preferentially and competes with tubulin at the tubulin binding site of MAPs.[5,14] Furthermore, we found that the interaction between tubulin and myosin is partially suppressed by PS-K. Thus, the present results on the influence of PS-K on actomyosin seem to support the hypothesis that PS-K might have a similar configuration to the tubulin molecule in part, and that this is important for the interaction with myosin and MAPs.

Non-muscle actins and skeletal muscle actin were considered to resemble each other closely in several biochemical properties such as molecular weight, amino acid composition, and activation of myosin ATPase activity.[6a,15] On the other hand, some differences between myosins of skeletal muscle and non-muscle cells have been reported. However, skeletal muscle F-actin was capable of forming a physiological complex with myosins from leukocytes, platelets, and macrophages, and of stimulating their ATPase activity in the same way as skeletal muscle myosin.[6a,16] The factor(s) in PS-K which inhibits actomyosin ATPase activity was extremely stable, as is the case with microtubule proteins,[17] because heat treatment and digestion with deoxyribonuclease (DNase) I, ribonuclease (RNase) A, proteases and glycosidases did not reduce its inhibitory effect (data not shown). The stability is consistent with the fact that PS-K given *p.o.* was effective against several tumors.[1a] The present results indicate that PS-K may affect the acto-myosin system as well as microtubules in immunocompetent cells.

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