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Effect of Protein-Bound Polysaccharide (PS-K) on Microtubule Proteins. III.[1] Some Properties of PS-K Inhibition of Microtubule Polymerization and the Site of Its Action

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A protein-bound polysaccharide (PS-K) suppressed glycerol-free microtubule polymerization in the same way as it suppressed the polymerization in a reassembly mixture containing glycerol. However, the extent of inhibition was enhanced in the absence of glycerol. Maximal inhibition reached about 85% (the extent of polymerization was 15% of the original level) in the standard assembly medium containing 100 mM KCl. The addition of PS-K increased the critical concentration for microtubule polymerization. Electron microscopy indicated that PS-K did not change the ultrastructure of microtubules. During incubation with PS-K, microtubule proteins retained their ability to polymerize, because the addition of taxol largely restored the inhibition by PS-K and the presence of an excess amount of microtubule-associated proteins (MAPs) or tubulin dimers also sequestered the inhibition to some extent. The extent of cancellation was greater by MAPs than by tubulin dimers.

Keywords——brain microtubule protein; polymerization; protein-bound polysaccharide; glycerol; electron microscopy; taxol; microtubule-associated protein; tubulin

Cytoplasmic microtubules are fibrous intracellular organella found in almost all eukaryotic cells. These organelles are formed by assembly of tubulin, a heterodimer which consists of two almost identical molecules with molecular weights close to 55000, and are known to be involved in various functions including mitosis, secretion, axonal transport, receptor activity, and maintenance of cell shape.[2] Besides tubulin dimers, microtubule proteins contain several other species called microtubule-associated proteins (MAPs) which promote tubulin polymerization.[3]

A protein-bound polysaccharide, PS-K, isolated from Basidiomycetes, has been shown to possess antitumor activity against various tumor cells.[4] PS-K is assumed to restore the depressed functions of lymphocytes or macrophages in tumor-bearing hosts, though the precise mechanism at the molecular level is not yet known.[4,5] These cells contain microtubule proteins. All mitotic spindles so far examined employ microtubules as a major fibrous component, and their assembly and disassembly participate in producing and regulating chromosome movements.[6] Colchicine and vinblastine, potent inhibitors of microtubule polymerization, have been reported to alter *in vitro* lymphocyte cap formation, blast transformation, and mitogen-induced deoxyribonucleic acid (DNA) synthesis.[7] Therefore, the state of microtubule assembly and disassembly may be important for the regulation of the response of various cells including lymphocytes and macrophages to various stimuli.

Recently, we found *in vitro* that PS-K partially inhibits the assembly of microtubules in reassembly buffer containing 1.7 M glycerol.[8] The experimental conditions were far from physiological. In this paper, we describe the characterization of the inhibitory action of PS-K on microtubule polymerization using glycerol-free microtubule proteins, and we propose that

the drug may bind to MAPs and tubulin dimers.

Materials and Methods

Materials——PS-K was purchased from Kureha Chemical Industry Co.; guanosine triphosphate (GTP) and 2-(*N*-morpholino)ethanesulfonic acid (MES) from Boehringer, pepstatin from Protein Research Foundation, and phosphocellulose from Whatman. Taxol was a generous gift from Drs. J. Douros and M. Suffness, National Cancer Institute, National Institutes of Health, Bethesda, MD, U.S.A. It was dissolved at 2 mM in dimethyl sulfoxide (DMSO) and stored at −20 °C. Other chemicals used were of reagent grade.

Preparation of Proteins——Glycerol-free microtubule proteins were prepared by three cycles of temperature-dependent polymerization and depolymerization. Microtubule proteins for the first cycle were isolated from porcine brains as described previously[9] and the second and third cycles were performed as described by Asnes and Wilson[10] with some modifications. The reconstituted microtubules after the first cycle were dispersed in cold buffer A [100 mM MES–KOH (pH 6.8), 0.5 mM $Mg(CH_3COO)_2$, and 1 mM ethyleneglycol-bis(2-aminoethyl ether)-*N*, *N*, *N'*, *N'*-tetraacetic acid (EGTA)] containing 2 μg/ml pepstatin by homogenization with a motor-driven Teflon-glass homogenizer and centrifuged at 39000 × *g* for 40 min at 4 °C. The supernatant was supplemented with 1/10 volume of a solution containing 25 mM GTP, 5 mM phosphoenolpyruvate, and 5 μg/ml pyruvate kinase and the mixture was incubated for 25 min at 37 °C. The solution was then centrifuged at 39000 × *g* for 40 min at 25 °C. The polymerization and depolymerization cycle was repeated once more.

Microtubule proteins in buffer A passed through a phosphocellulose column equilibrated with the same solution. Tubulin was recovered as the first protein fraction at the exclusion limit.[9]

MAPs were prepared by the method of Herzog and Weber[3a] with some modifications. After depolymerization of reconstituted microtubules from the third cycle in cold buffer A containing 0.8 M NaCl and 0.5% 2-mercaptoethanol, the suspension was incubated for 4 min in a boiling water bath and cooled rapidly in ice. The denatured protein was removed by centrifugation at 20000 × *g* for 20 min at 4 °C and the supernatant, containing MAPs, was dialyzed against buffer A. The purified tubulin fraction showed no detectable band other than tubulin on 10% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS).[11] On the other hand, the MAPs fraction was mainly composed of high-molecular-weight proteins and tau proteins.

Microtubule Polymerization——Microtubule polymerization was assayed by measuring turbidity or by viscometry. The standard reaction mixture consisted of 80 mM MES–KOH (pH 6.5), 1 mM $Mg(CH_3COO)_2$, 0.5 mM EGTA, 1 mM GTP, and 2 mg microtubule proteins in a final volume of 1 ml. Polymerization was monitored by recording the change in absorbance at 350 nm using a Jasco UVIDEC-410 spectrophotometer equipped with an automatic recorder at 37 °C in a thermostatically regulated sample chamber. The extent of polymerization was expressed in terms of turbidity increase at 30 min after the start of incubation. Viscosity measurements (Ostwald-type viscometer) were also performed to monitor microtubule polymerization at 37 °C.

Electron Microscopy——For thin-section electron microscopy, microtubules in the presence or absence of PS-K were sedimented at 39000 × *g* for 40 min at 25 °C, fixed in 2% glutaraldehyde, post-fixed in 1% OsO_4, and embedded in Epon 812.[12] The sections were examined with a Hitachi HU-12A electron microscope.

Protein Concentration——Protein was determined by the method of Lowry *et al.*[13] with bovine serum albumin as a standard.

Results

The purification methods of microtubule proteins are based on the temperature dependence of polymerization and depolymerization. It was previously reported that a procedure in which a reassembly solution containing glycerol was used had been applied to isolate microtubule proteins from porcine brains.[8] Since glycerol has been demonstrated to alter the characteristics of microtubule polymerization, although it shows stabilizing and polymerization-enhancing effect on microtubule proteins,[14] we examined the effect of PS-K on the polymerization using glycerol-free microtubule proteins (Fig. 1). When microtubule polymerization was measured by viscometry, PS-K inhibited both the initial velocity and extent of the polymerization in a dose-dependent manner. PS-K *per se* showed no change of viscosity during incubation with GTP. The rate and extent of inhibition seemed to be greater than in the case of a reassembly solution containing glycerol. The same phenomena were found when microtubule polymerization was assayed by measuring turbidity.

Figure 2 shows the dose-response effect of PS-K on microtubule polymerization with or

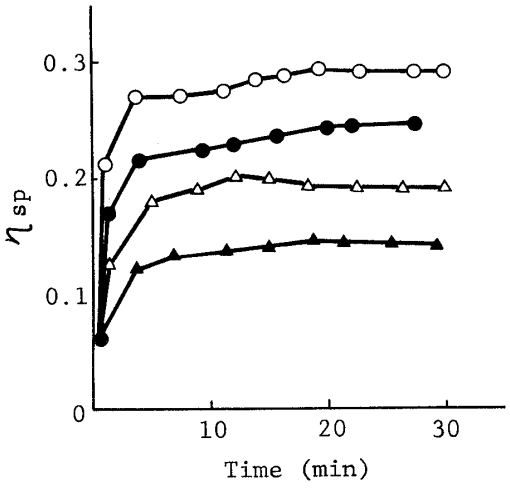

Fig. 1. Effect of PS-K on Microtubule Polymerization

Reassembly medium consists of 80 mM MES–KOH (pH 6.5), 1 mM $Mg(CH_3COO)_2$, 0.5 mM EGTA, and 1 mM GTP. The concentration of microtubule proteins was 1.63 mg/ml. ○, (−); ●, 0.1 mg/ml; △, 0.2 mg/ml; ▲, 0.4 mg/ml PS-K.

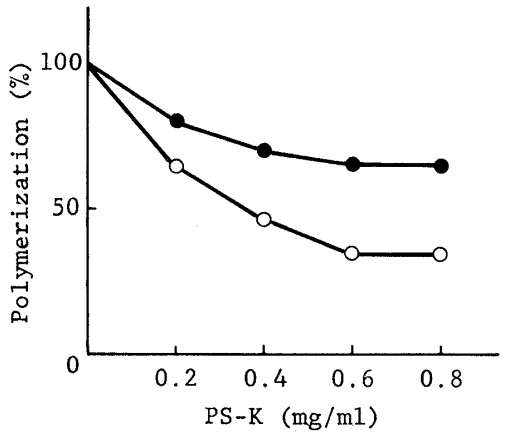

Fig. 2. Effect of Glycerol on the Inhibitory Action of PS-K

Microtubule polymerization was measured under the standard conditions modified to contain the concentrations of PS-K indicated. Polymerization was expressed terms of the absorbance at 350 nm after the initial 30 min and the values in the absence of PS-K were taken as 100%. ○, (−) glycerol; ●, 1.7 M glycerol.

without glycerol. The addition of 1.7 M glycerol to the reassembly mixture reduced the inhibitory effect of PS-K. The concentration of PS-K which was required for half-maximal inhibition in the presence or absence of glycerol was about 0.2 mg/ml. Both inhibitions reached a plateau when over 0.6 mg/ml of PS-K was added to the reassembly mixture containing 2 mg/ml of microtubule proteins.

It is well established that high concentrations of monovalent and calcium ions suppress microtubule assembly *in vitro* and that calcium ions are involved in the regulation of the assembly and disassembly of the microtubule system *in vivo*.[15] The percentage of microtubules which polymerized in the presence of added KCl is plotted as a function of KCl concentration (Fig. 3). When the polymerization was carried out under our experimental conditions, the addition of PS-K (0.6 mg/ml) reduced the extent of polymerization to about 35% of the original level in the absence of the drug as shown in Fig. 2. Further addition of KCl (100 mM) potently suppressed the extent of polymerization in the presence of PS-K, whereas it had little effect on the extent without PS-K (Fig. 3). Finally, the inhibition by PS-K reached more than 80% in the standard reaction mixture containing 100 mM KCl. Similar phenomena were observed when glycerol (1.7 M) was contained in the reassembly medium. In the presence of PS-K, the concentration of added KCl required to give complete inhibition is lower than that in its absence. The enhancement of inhibitory effect was also found when NaCl was used instead of KCl. Although calcium ions at the mM level suppressed microtubule polymerization *in vitro*, the extent of inhibition by PS-K was almost the same over a wide range (0—2.5 mM) of calcium ion concentration.

It has been demonstrated that lowering the concentration of MAPs raises the critical concentration for microtubule polymerization.[16] Figure 4 shows the effect of PS-K on the critical concentration. Under the conditions tested, PS-K (0.6 mg/ml) increased the critical concentration of microtubule proteins from 0.3 to 1 mg/ml. The inhibition was almost complete at low concentrations of microtubule proteins. However, microtubule assembly was found at 0.3—1 mg/ml of microtubule proteins when the ratio of PS-K to microtubule proteins was held constant at 0.33 (w/w).

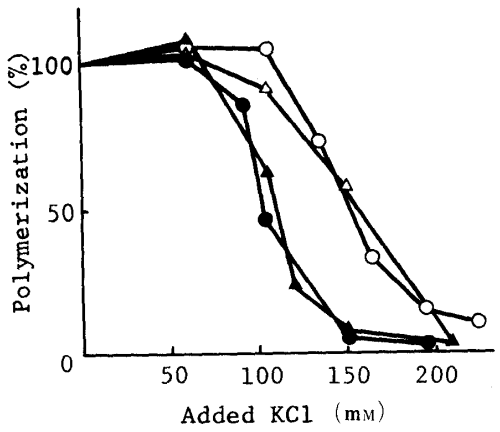

Fig. 3. Effect of KCl on the Inhibitory Action of PS-K

Microtubule polymerization was measured under the standard conditions modified to contain the concentrations of KCl indicated. The extents of polymerization in the absence of added KCl were taken as 100%. ○, no addition; ●, 0.6 mg/ml PS-K; △, 1.7 M glycerol; ▲, 0.6 mg/ml PS-K + 1.7 M glycerol.

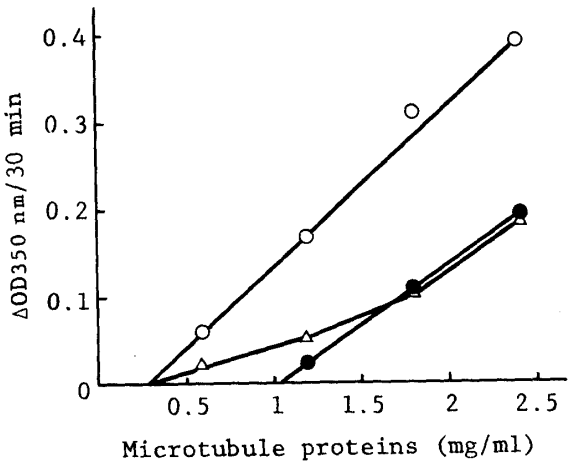

Fig. 4. Effect of PS-K on the Critical Concentration for Microtubule Polymerization

Various amounts of a microtubule preparation were polymerized in the presence or absence of PS-K. ○, (−) PS-K; ●, 0.6 mg/ml PS-K; △, PS-K microtubule proteins = 0.33 (w/w).

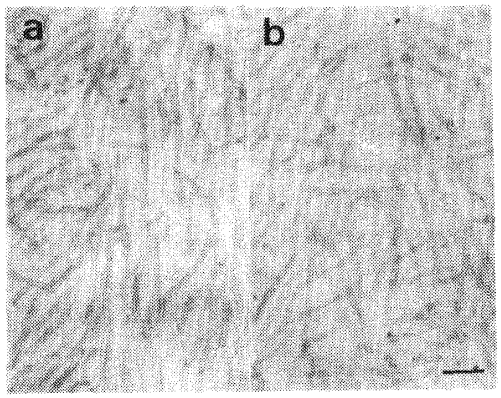

Fig. 5. Electron Micrographs of Microtubules Polymerized in the Presence or Absence of PS-K

Microtubule proteins (2 mg/ml) were polymerized at 37 °C for 30 min. a, (−) PS-K; b, 0.6 mg/ml PS-K. Bar; 100 nm.

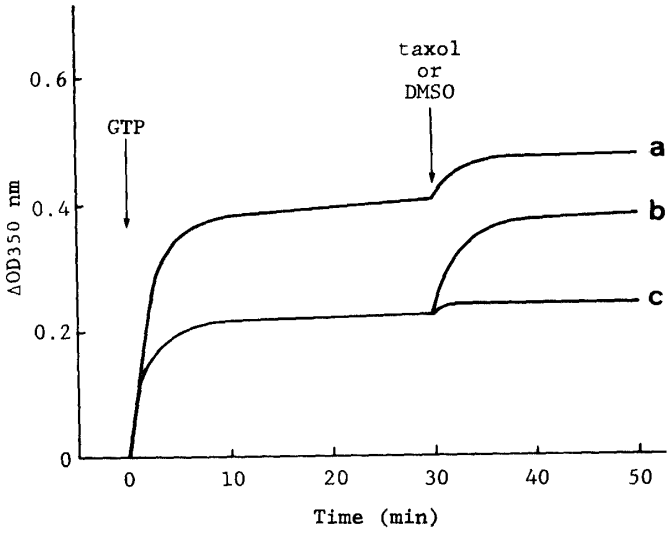

Fig. 6. Release of PS-K Inhibition by the Addition of Taxol

Microtubule polymerization was measured under the standard conditions. The concentrations of PS-K and taxol were 0.4 mg/ml and 20 μM, respectively. a, microtubule proteins + taxol (10 μM); b, (microtubule proteins + PS-K) + taxol; c, (microtubule proteins + PS-K) + DMSO. Materials indicated within parentheses were preincubated together before the addition of the remainder.

Polymerized forms of tubulin were examined by thin-section electron microscopy (Fig. 5). After incubation of microtubule proteins with or without PS-K, microtubules were pelleted by centrifugation at 39000 × *g* for 40 min at 25 °C and the pellets were fixed in glutaraldehyde. It does not seem likely that PS-K affected the structure of microtubules under our experimental conditions.

Taxol, a natural product from *Taxus brevifolia*, has been reported to enhance microtubule assembly and to stabilize microtubules both *in vitro* and *in vivo*.[17] In the presence of taxol, microtubules polymerized without GTP or MAPs and, further, the drug decreased the

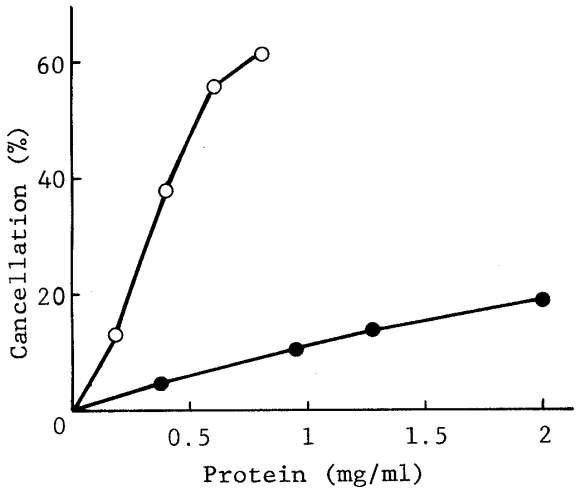

Fig. 7. Restoration of the Inhibitory Action of PS-K by the Addition of MAPs

Experimental conditions were the same as in Fig. 2. The concentration of PS-K was 0.6 mg/ml. Cancellation was defined as follows: (1-extent of inhibition with exogenous MAPs or tubulin dimers/extent of inhibition without exogenous MAPs or tubulin dimers) × 100. ○, MAPs; ●, tubulin dimers.

critical concentration to less than 0.015 mg/ml, while the concentration in its absence was about 0.3 mg/ml, as shown in Fig. 4. When turbidity development reached a plateau during incubation of microtubule proteins (2 mg/ml) with GTP (1 mM) for 30 min at 37 °C, the addition of taxol (20 μM) induced further tubulin polymerization (Fig. 6, curve a). On the other hand, the addition of taxol to the reassembly mixture containing PS-K (0.6 mg/ml) remarkably increased the turbidity, though its final extent was lower than that in the absence of PS-K (curve b). DMSO, the solvent for taxol, had little effect on the turbidity change (curve c). Polymerizability by taxol was also found when the reassembly was conducted in the presence of glycerol.

The above results led us to carry out experiments to investigate whether PS-K binds to tubulin dimers or MAPs. Figure 7 shows the effect of excess amounts of tubulin dimers or MAPs on the inhibition by PS-K. Original microtubule proteins were mixed with exogenous tubulin dimers or MAPs prior to the addition of PS-K and incubated for 5 min at 37 °C. After mixing with PS-K (0.6 mg/ml), microtubule polymerization was initiated by the addition of GTP. The addition of tubulin dimers or MAPs could reduce the inhibitory action of PS-K in a dose-dependent manner to some extent under conditions where microtubule assembly was inhibited about 60% by PS-K. The extent of cancellation by MAPs was greater than that by tubulin dimers.

Discussion

The present results show that PS-K inhibits microtubule polymerization under various conditions. PS-K shifts the equilibrium in favor of tubulin dimers. The extent of inhibition was enhanced when glycerol was excluded in the assay of polymerization (Fig. 2). We reported recently that the concentrations of PS-K required for half-maximal and maximal inhibition were 0.3 mg/ml and over 0.7 mg/ml, respectively.[8] These values were greater than those obtained in this experiment. This discrepancy may be ascribed to the difference in the method of preparation used. On the addition of KCl at a final concentration of 100 mM to the standard reaction mixture (ionic strength; about 0.15), PS-K further inhibited microtubule polymerization, and the maximal inhibition reached about 85% (the extent of polymerization was about 15% of that in the absence of drug) (Fig. 3). PS-K seemed to suppress microtubule polymerization effectively under nearly physiological conditions.

As shown in Figs. 6 and 7, the addition of taxol or excess amounts of MAPs cancels out the inhibitory effect of PS-K to some extent, indicating that microtubule proteins survive during incubation with the drug and the interaction between microtubule proteins and PS-K

is reversible. The striking feature of the inhibition is the ability of both MAPs and tubulin dimers to reverse it. Microtubule preparation is composed of 75% tubulin dimers and 25% MAPs on SDS–polyacrylamide gel electrophoresis, as reported previously.[3c,10] MAPs (0.5 mg/ml) added in an amount equal to the original MAP content in the microtubule proteins caused a considerable restoration of microtubule assembly (about 45%), while the addition of tubulin dimers (1.5 mg/ml) caused a slight restoration (about 15%). Although the mechanism(s) by which PS-K interferes with the functions of microtubule proteins is unknown at present, one possibility is that PS-K interacts with MAPs preferentially and competes with tubulin dimers at the tubulin binding sites of MAPs.

Recently, colchicine and podophyllotoxin have been shown to inhibit taxol-induced polymerization through binding to tubulin dimers when they are preincubated with microtubule proteins prior to the addition of taxol, whereas microtubules polymerized by taxol are generally resistant to depolymerization by these drugs.[18] However, taxol induced polymerization even when PS-K was preincubated with microtubule proteins (Fig. 6). Therefore, the mode of inhibition by PS-K appears to be different from that by colchicine or podophyllotoxin.

Vinca alkaloids have been shown not only to block the formation of microtubules but also to induce the formation of various oligomeric structures such as rings, spirals, and paracrystals.[19] Electron microscopy revealed that PS-K does not affect the structure of microtubules in this way (Fig. 4).

A number of antimitotic reagents including colchicine, podophyllotoxin, griseofulvin, ansamitocins, maytansinoids, and Vinca alkaloids are thought to mediate their antitumor effects through binding to microtubule proteins.[20] These drugs are also known to inhibit microtubule assembly in the presence or absence of glycerol at low concentrations which are approximately the same as the concentration of tubulin dimers. Recently, a polysaccharide from sea urchin egg, mainly composed of fucose, has been reported to inhibit completely microtubule assembly when the ratio of the factor to microtubule proteins is over 0.03 (w/w), and the inhibitor is sensitive to digestion by glycosidase mixture containing mannosidases.[21] PS-K, a protein-bound polysaccharide which is mainly composed of glucose, also shows an inhibitory action on microtubule assembly but high concentrations of the drug are required for saturation of the inhibition. In addition, treatment of PS-K with a glycosidase mixture containing glucosidases did not reduce the inhibitory effect.[8]

It has been shown that biochemical differences among tubulin species from a variety of sources are minor and MAPs are contained in several cultured cells.[22] Immunocytochemical studies suggest that MAPs from brain tissue are associated with the mitotic spindle and the filamentous cytoplasmic networks in various tissue and cell lines.[22a,b,23] Thus, PS-K may affect assembly and disassembly of microtubules in lymphocytes and macrophages if the drug is incorporated into these cells.

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