## Communications to the Editor

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# INHIBITION OF ADENOSINE-5'-TRIPHOSPHATASE ACTIVITY IN MICROTUBULE PROTEIN PREPARATION BY PS-K

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A protein-bound polysaccharide, PS-K, exhibited an inhibitory action on the ATPase activity of microtubule proteins in a concentration-dependent manner. The extent of inhibition was greater with $Mg^{2+}$ than with $Ca^{2+}$. Heat treatment at 100°C and digestion by DNase 1, RNase A, several proteases, and a glycosidase mixture did not affect the inhibitory effect of PS-K.

KEYWORDS——ATPase; brain microtubule protein; inhibitory factor; PS-K; stable factor

A protein-bound polysaccharide (PS-K) isolated from Basidiomycetes, has been reported to show antitumor activity against various experimental tumors including sarcoma-180, hepatoma AH-13, Ehrlich tumor, and 3-methylcholanthrene-induced fibrosarcoma.[1] Although the antitumor action of the drug is supposed to be mediated by some defense mechanisms of the host, mainly consisting of immune response, the precise mechanism in the cellular components remains unknown. Recently, we found that PS-K suppressed brain microtubule polymerization in a dose-dependent manner.[2] Cytoplasmic microtubules are thought to perform various functions such as mitosis, secretion, receptor activity, axonal transport, and maintenance of cell shape.[3] Some of these processes presumably generate force by transforming chemical energy (ATP) into mechanochemical work. In fact, recent evidence has indicated that microtubule preparations isolated from brain by cycles of polymerization and depolymerization contain a unique ATPase.[4] Thus, it is of interest to determine whether PS-K affects the ATPase activity in microtubule proteins.

Microtubule proteins were prepared from porcine brain by three cycles of the temperature-dependent polymerization and depolymerization scheme described by Ihara *et al.*[4a] The purified proteins were stored at -80°C until use. Protein was determined by the method of Lowry *et al.*[5] using bovine serum albumin as a standard.

ATPase activity was determined by measuring the amount of inorganic phosphate, using the colorimetric procedure.[6] The reaction mixture consisted of 60 mM 2-(N-morpholino)ethanesulfonic acid (MES)-KOH (pH 6.5), 5 mM 2-mercaptoethanol, 12.5% glycerol, 2 mM ATP, 0.5 mg/ml of microtubule proteins, and either 2 mM $Mg(CH_3COO)_2$ or 5 mM $CaCl_2$ in a final volume of 0.4 ml. The reaction was started by adding ATP

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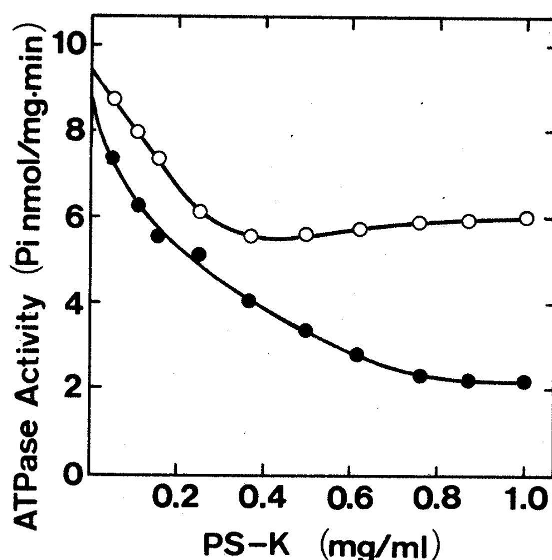


Fig. 1. Inhibition of ATPase Activity of Microtubule Proteins by PS-K
●, 2 mM $Mg(CH_3COO)_2$; ○, 5 mM $CaCl_2$

and stopped after incubation for 30 min at 37°C by adding 0.4 ml of 20% trichloroacetic acid. The rate of ATP hydrolysis was determined to be linear over this period of time.

The PS-K used was a generous gift from Kureha Chemical Industry Co. The glycosidase mixture, purchased from Seikagaku Kogyo Co., contained α-mannosidase, β-mannosidase, α-glucosidase, β-glucosidase, α-galactosidase, β-galactosidase, α-L-fucosidase, β-xylosidase, α-N-acetylglucosaminidase, β-N-acetylglucosaminidase, α-N-acetylgalactosaminidase, and β-N-acetylgalactosaminidase. DNase 1, RNase A, α-chymotrypsin, papain, and proteinase K were purchased from Sigma Chemical Co., trypsin from Worthington, and pronase E from Kakenkagaku Co. Other chemicals used were reagent grade.

It was first found in this experiment that PS-K inhibited $Mg^{2+}$- or $Ca^{2+}$-ATPase activity associated with microtubule proteins (Fig. 1). The extent of inhibition increased with increasing amounts of PS-K added, though the inhibition in the presence of $Ca^{2+}$ was somewhat weaker than with 0.4 mg/ml PS-K. In the presence of $Mg^{2+}$, maximal inhibition reached about 25% of the original level, while the value in the presence of $Ca^{2+}$ was about 60%. The concentrations of PS-K required for half-maximal inhibition were 0.18 mg/ml in the presence of $Mg^{2+}$ and 0.14 mg/ml in the presence of $Ca^{2+}$. The inhibition by PS-K was not considered to be caused by contaminating ions or low molecular weight components, because the PS-K prepared after dialyzing against 20 mM MES-KOH (pH 6.5) for 48 h (molecular weight cutoff: 6,000-8,000) retained the inhibitory effect. When GTP, which was essential for microtubule polymerization, was used as a substrate, PS-K inhibited $Mg^{2+}$-GTPase activity as it did ATPase activity.

The concentration of $Ca^{2+}$ required for maximal activation of the ATPase activity of microtubule proteins was reported to be unphysiologically high (mM order)[4a,b] and, further, the final extent of inhibition by PS-K was greater with $Mg^{2+}$ than with $Ca^{2+}$. Thus, PS-K may affect $Mg^{2+}$-ATPase activity.

Some properties of PS-K are summarized in Table I. The inhibitory action of PS-K on the $Mg^{2+}$-ATPase activity of microtubule proteins was heat-stable, retaining its activity even when incubated for 2 h at 100°C. Therefore, heat treatment was used as a tool for inactivation of enzyme digestion. The preparation of PS-K treated with DNase 1 or RNase A for 1 h at 37°C did not lose its inhibitory effect. Since the active substance of PS-K has been reported to be a protein-bound polysaccharide which was mainly composed of glucose,[1a,7] we examined whether or not the inhibition takes place after treatment of PS-K with various proteases and

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Table I. Inhibitory Activity of PS-K on $Mg^{2+}$-ATPase Activity after Various Treatments

| Treatment | | Relative activity** (%) |
|---|---|---|
| None | | 37 |
| 100°C 0.5 h | | 39 |
| 1 h | | 38 |
| 2 h | | 43 |
| DNase 1 (0.1 mg/ml) 37°C 1 h | | 40 |
| RNase A (0.1 mg/ml) 37°C 1 h | | 40 |
| Trypsin (0.1 mg/ml) 37°C 1 h | | 37 |
| α-chymotrypsin (0.1 mg/ml) 37°C 1 h | | 43 |
| Papain (0.1 mg/ml) 37°C 1 h | | 41 |
| Pronase E (0.1 mg/ml) 37°C 1 h | | 40 |
| Proteinase K (0.1 mg/ml) 37°C 1 h | | 43 |
| Glycosidase mixture (0.1 mg/ml) 37°C | 8 h* | 36 |
| | 24 h | 40 |

After incubating of 5 mg/ml PS-K with the indicated treatment, the samples were heated at 100°C for 10 min to inactivate enzyme activities. *PS-K dissolved in 10 mM acetate buffer (pH 4) was treated with the glycosidase mixture and then dialyzed against 10 mM MES-KOH (pH 6.5). **Relative activity represents the ratio of $Mg^{2+}$-ATPase activity with PS-K (final concentration: 0.5 mg/ml) to the activity without it, as a percentage.

glycosidases. The inhibitory effect was not destroyed by incubation with trypsin, α-chymotrypsin, papain, pronase E, proteinase K, or a glycosidase mixture containing glucosidases. In addition, treatment with trypsin, α-chymotrypsin, or proteinase K after incubation of PS-K with the glycosidase mixture for 24 h did not reduce the inhibitory activity. That these characteristics of PS-K were very similar to those required to suppress microtubule polymerization is indicated by the fact that the factor(s) in PS-K which inhibits polymerization was heat-stable and its action was not lost in the various treatments, as described previously.[2)] This stability is also in agreement with the fact that the antitumor effect of PS-K was effective against several tumors by oral administration.[1a)]

The effect of PS-K is considered to restore the depressed functions of lymphocytes or macrophages in tumor-bearing hosts and, further, microtubule proteins are contained in these cells.[1a,8)] When brain microtubule proteins were separated into microtubule-associated proteins (MAPs) and tubulin by phosphocellulose column chromatography, the ATPase activity was recovered in the MAPs fraction.[4a,c,9)] Recently, we found that PS-K may interact with both MAPs and tubulin (manuscript in preparation). Immunofluorescence staining of various cultured cells using antibodies to MAPs reveals that cytoplasmic and mitotic-spindle microtubules are coated all along their lengths with associated proteins very similar to those

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found in brain.[10] However, it is not certain at present that microtubule proteins other than those in brain involve an ATPase. The present experiments suggest that stable factor(s) contained in PS-K suppresses $Mg^{2+}$-ATPase activity as well as microtubule polymerization.

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