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Promotion of ATP and S-140 to ribosome inactivation with camphorin, cinnamomin, and other RNA *N*-glycosidases[1]

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KEY WORDS ribosome-inactivating proteins; immunotoxin; rRNA; camphorin; cinnamomin; luffin; γ-momorcharin; ricin; trichosanthin

AIM: To study the effect of ATP and extra-ribosomal factors (S-140) on type Ⅰ and type Ⅱ RNA *N*-glycosidases in inactivating ribosome. **METHODS**: The activity of ATP and S-140 was determined by characterization of R-fragment in gel. An improved two-step method of cell-free protein synthesis system was used to quantitate the requirements of S-140 in ribosome inactivation. **RESULTS**: IC_{50} ratios of camphorin, γ-momorcharin, luffin S, luffin A, trichosanthin (type Ⅰ); and ricin, ricin A-chain; cinnamomin, cinnamomin A-chain (type Ⅱ) between the absence and presence of ATP and S-140 were 3108, 151, 51, 45, 15; and 47, 7, 26, 12, respectively. **CONCLUSION**: The ribosome-inactivating activity of type Ⅱ ribosome-inactivating proteins, including intact protein and its A-chain, was promoted by ATP and S-140. Camphorin showed a significant difference from cinnamomin in need of ATP and S-140 for such promoting.

Ribosome-inactivating proteins (RIP) are specific RNA *N*-glycosidases that catalyze irreversible damages to ribosomes by hydrolizing the glycosidic bond of a unique, highly conserved adenosine residue in the largest ribosomal RNA: A_{4324} in rat liver[1,2]. The depurination of 28S rRNA made the damage site very susceptible to acid-aniline cleavage and released the diagnostic RNA fragment (R-fragment). RIP have been used in construction of therapeutic agents such as anticancer immunotoxins, neuronal transmitter-toxins, and anti-HIV drugs.

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Camphorin (single-chain) and cinnamomin (two-chain) are two novel RNA *N*-glycosidases purified in our laboratory from the seeds of *Cinnamomum camphora* Nees *et* Eberm. Beside the RNA *N*-glycosidase activity[3], camphorin has the activity to cleave supercoiled double-stranded DNA into nicked and linear form[4], and a third, which is by now the only case in RIP, the superoxide dismutase (SOD) activity[5].

Earlier evidence indicated that tritin[6], gelonin[7,8], and several other type Ⅰ RIP[9-11] require the presence of ATP and factors of rabbit reticulocyte post-ribosomal supernatant for inactivation of the isolated ribosme that otherwise was notably resistant to these RIP. The present study was to extend the above studies on the co-factor requirement to as many RIP as available, including type Ⅱ RIP, which had not been found of co-factors requirement for promoting their activities, and some unique type Ⅰ RIP such as camphorin having multiple functions and γ-momorcharin with the smallest molecular weight identified up to now.

MATERIALS AND METHODS

Materials Camphorin (M_r 23×10^3) and cinnamomin (M_r 64.5×10^3) were prepared from the seeds of *Cinnamomum camphora* as described[5]. Camphorin was further purified and the improved procedures will be published elsewhere. The reduced cinnamomin by treatment with 2-mercaptoethanol was used as cinnamomin A-chain in this study. Gelonin (M_r 30.5×10^3) was a gift of Prof F Stirpe, Dipartimento di Patologia sperimentale dell'Universita di Bologna, Italy. Luffin A (M_r 28×10^3) and luffin S (M_r 14.8×10^3) were kindly donated by Prof ZHANG Zu-Chuan, Shanghai Institute of Biochemistry, Chinese Academy of Sciences. γ-Momorcharin (M_r 11.5×10^3) was generously given by Prof JIN Shan-Wei, Shanghai Institute of Organic Chemistry, Chinese Academy of

Sciences. Trichosanthin (M_r 27 × 10^3) was generously supported by Prof KE Yi-Bao, Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Poly (U), β-glycerophosphate, ricin, and ricin A-chain were purchased from Sigma Chemical Co, USA. *L*-[^{3}H]Phenylalanine was obtained from Shanghai Institute of Nuclear Research, Chinese Academy of Sciences. All RIP used were electrophoresis analysed.

Preparation of ribosomes and extra-ribosomal factors Rat liver 80S ribosomes were prepared by the method of Spedding[12] with a modification that high-salt (KCl 0.5 mol·L^{-1}) washing step was performed twice. Gel filtered post-ribosomal supernatant (S-140) was prepared according to the method of Brigotti *et al*[10].

Assay of SOD activity The SOD activity was assayed by negative activity staining in polyacrylamide gel[13].

Assay of RNA *N*-glycosidase activity Treatment of ribosomes with RIP and electrophoretic identification of R-fragment were performed by the method of Endo *et al*[2]. Salt-washed 80S ribosomes (20 pmol) in 50 μL of buffer A (Tris/HCl 15 mmol·L^{-1}, pH 7.3, KCl 100 mmol·L^{-1}, $MgCl_2$ 2 mmol·L^{-1}, β-glycerophosphate 10 mmol·L^{-1}) were incubated at 37 ℃ for 15 min with RIP in the presence or absence of ATP 1 mmol·L^{-1} and 3 μL S-140 (8 μg as non-hemoglobin protein). Secondly, the rRNA were extracted and treated with acid aniline at 60 ℃ for 15 min, and then analysed by a urea-denaturing polyacrylamide gel (3.5 %), the RNA bands were visualized by ethidium bromide staining.

Protein synthesis *in vitro* Experimental procedures of the poly(U) translation assay and of the two-step assay for quantitatively testing the requirement of ATP and S-140 by RIP were improved based on the method of Carnicelli *et al*[9]. Briefly, ribosomes (20 pmol) were preincubated with RIP at 32 ℃ for 15 min in 50 μL of buffer A as the same treatment in the first step of the assay of RNA *N*-glycosidase activity. At the end of preincubation, ribosomes were recovered by centrifugation (35 000 × *g*, at 4 ℃ for 90 min, using a RPR 20-3 rotor, SCR 20BA centrifuge, Hitachi) and then resuspended with buffer B (Tris/HCl 80 mmol·L^{-1}, pH 7.3, KCl 120 mmol·L^{-1}, $MgCl_2$ 7 mmol·L^{-1}, dithioerythritol 2 mmol·L^{-1}, β-glycerophosphate 10 mmol·L^{-1}). Poly(U) translation activity was assayed in 100 μL of buffer B containing ATP 1 mmol·L^{-1}, GTP 0.2 mmol·L^{-1}, 'pH 5 enzyme' 20 μL, S-100 20 μL, phosphocreatine 1 μmol·L^{-1}, phosphokinase 4 μg creatin, poly (U) 80 μg, ribosomes 20 pmol and *L*-[^{3}H]phenylalanine 185 kBq. After incubation at 37 ℃ for 30 min, the hot-acid-insoluble radioactivity was measured and IC_{50} (concentration causing 50 % inhibition of protein synthesis) was calculated.

RESULTS AND DISCUSSION

The camphorin exhibited the SOD activity (Fig 1) and the supercoil-dependent endonuclease activity. These results further confirmed our previously reports[4,5].

Fig 1. Superoxide dismutase activity of camphorin. The protein samples were separated by electrophoresis in 10 % polyacrylamide gel. A) Camphorin (2 μg) was stained by silver. B) SOD activity of camphorin was assayed by negative activity staining.

In contrast with the earlier work of our lab which showed the IC_{50} of intact cinnamomin, cinnamomin A-chain, and camphorin were 9.7, 1.0, and 0.098 nmol·L^{-1}, respectively in a rabbit reticulocyte lysate system in which the extra-ribosomal factors were retained on ribosomes[3], firstly we analysed RNA *N*-glycosidase activity of these RIP to inactivate ribosomes that were isolated through high-salt washing twice by which the remained extra-ribosomal factors were eliminated (Fig 2).

Salt - washed ribosomes were strikingly

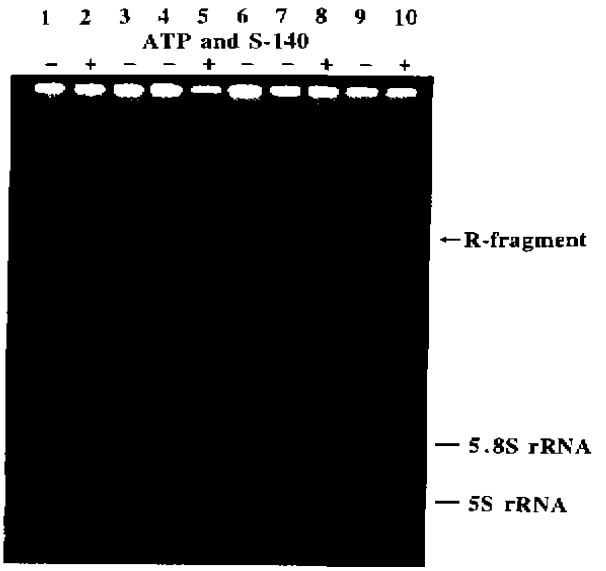

Fig 2. Effects of ATP and S-140 on the RNA *N*-glycosidase activities of camphorin and cinnamomin A-chain. Lane 1 and 2, control; lane 3, cinnamomin A-chain 2.0 nmol·L^{-1}; lane 4 and 5, cinnamomin A-chain 0.1 nmol·L^{-1}; lane 6, camphorin 1.0 μmol·L^{-1}; lane 7 and 8, camphorin 20.0 nmol·L^{-1}; lane 9 and 10, gelonin 2.0 nmol·L^{-1}. The arrow indicates the R-fragment released as a consequence of ribosome-inactivating protein action after acid-aniline treatment.

resistant to the action of camphorin even at a concentration higher than its IC_{50} level (20.0 nmol·L^{-1}), and such resistance could not be overcome until the amount of this RIP was increased up to more than 0.5 μmol·L^{-1}. However, when ATP and S-140 were added, a promoting effect to inactivate ribosome was evidently observed for camphorin at 20.0 nmol·L^{-1}. This phenomenon was similar with gelonin that required the co-factors to inactivate ribosome. Compare with the case of camphorin, salt-washed ribosomes were still susceptible to the action of cinnamomin A-chain at a concentration around its IC_{50}. However, at 0.1 nmol·L^{-1}, lower than its IC_{50}, ATP and S-140 must be added could the inactiviting activity of cinnamomin A-chain exhibit. Therefore, these data demonstrated that in order to inactivate salt-washed ribosomes, the presence of ATP and S-140 was required for camphorin, whereas it did not need for cinnamomin A-chain at the concentration around its IC_{50}. However, the ribosome-inactivation of cinnamomin A-chain could also be promoted at a concentration lower than its IC_{50}. The present data revealed that the ribosome-inactivating activity of type Ⅱ RIP A-chain can be promoted by ATP and S-140.

In order to quantitatively investigate the function of ATP and S-140 for RIP in inactivating ribosome, an improved two-step method of *in vitro* protein synthesis was employed. Ribosomes preincubated were recovered for removing RIP completely and the damage of the treated ribosome was assayed by polyphenylalanine synthesis. IC_{50} of low-toxic type Ⅰ RIP, such as camphorin and γ-momorcharin was notably lowered in the presence of ATP and S-140, while the addition of ATP and S-140 could only modestly promote the ribosome-inactivating activity of ricin, cinnamomin, and high-toxic type Ⅰ RIP — trichosanthin, luffin A, and luffin S. (Tab 1)

Tab 1. IC_{50} of RIP assayed for the inactivation of ribosomes in the absence and presence of gel-filtered 'S-140' and ATP[x].

RIP	IC_{50}/nmol·L^{-1} 'S-140' and ATP absent (a)	'S-140' and ATP present (b)	Ratio (a/b)
(Type Ⅰ)			
Camphorin	497 (377 – 617)	0.162 (0.117 – 0.207)	3108
γ-Momorcharin	71 (54 – 87)	0.472 (0.397 – 0.551)	151
Luffin S	63 (53 – 74)	0.84 (0.67 – 1.01)	51
Luffin A	3.60 (2.63 – 4.58)	0.084 (0.062 – 0.105)	45
Trichosanthin	0.371 (0.316 – 0.425)	0.0253 (0.0211 – 0.0288)	15
(Type Ⅱ)			
Cinnamomin	15.2 (12.9 – 17.6)	0.59 (0.48 – 0.69)	26
Cinnamomin A-chain	0.74 (0.63 – 0.85)	0.062 (0.051 – 0.072)	12
Ricin	11.8 (14.6 – 20.9)	0.251 (0.181 – 0.320)	47
Ricin A-chain	0.233 (0.194 – 0.265)	0.033 (0.024 – 0.042)	7

[x]Preincubation with 'S-140' and ATP had no effect on control phenylalanine incorporation. (n = 3 – 5 experiments).

The results presented here showed that ATP and extra-ribosomal factors S-140 were required for promotion to ribosome inactivation by all RIP tested, provided further evidences for the similarity between camphorin and gelonin, and supported the viewpoint that ATP and S-140

promoted the activity of type Ⅱ RIP in inactivating ribosome. Since the IC_{50} ratio of camphorin in the abesnce and presence of ATP and S-140 was far bigger than that of cinnamomin, camphorin could be easily distinguished from cinnanmomin in need of ATP and S-140 for promoting its ribosome-inactivating activity. This result confirmed our early report that camphorin itself exhibited RNA *N*-glycosidase activity[3].

Brigotti *et al*[10] reported that ATP and S-140 tRNA could not promote the ribosome-inactivating activities of trichosanthin and luffin. We proposed that the different findings might be due to the use of different assay systems. Moreover, the unfractionated co-factors (S-140) we used had about ten-fold promoting activity higher than that of crude S-140 tRNA[8,11]. It was difficult to interpret why the ribosome inactivation of high-toxic RIP could be promoted by ATP and S-140. The demostration of the widespread occurrence of RIP-promoting activity of ATP and S-140 and the new revealed promoting effects of co-factors on high-toxic RIP, especially on the A-chain of type Ⅱ RIP, for their ribosome-inactivation provided new challenges to the study in this field.

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ATP 和 S-140 促进克木毒蛋白、辛纳毒蛋白和其它 RNA *N*-糖苷酶失活核糖体的作用[1]

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关键词 核糖体失活蛋白; 免疫毒素; rRNA; 克木毒蛋白; 辛纳毒蛋白; 丝瓜毒蛋白; γ-苦瓜子毒蛋白; 蓖麻毒蛋白; 天花粉毒蛋白 ATP S-140

目的: 研究 ATP 和 S-140 对Ⅰ型和Ⅱ型核糖体失活蛋白(RIP)失活核糖体的影响. **方法**: 采用凝胶电泳分析特征 RIP 作用片断(R-片断), 以及改进的二步法标记苯丙氨酸外源 poly (U)翻译体系, 定量检测外加因子对 RIP 失活核糖体作用的影响. **结果**: ATP 和 S-140 对所有用于检测的Ⅰ型和Ⅱ型 RIP 失活核糖体的功能均表现出不同程度的促进作用. 对于Ⅰ型 RIP 中克木毒蛋白、天花粉毒蛋白、γ-苦瓜子毒蛋白、丝瓜毒蛋白 A、丝瓜毒蛋白 S 和Ⅱ型 RIP 中蓖麻毒蛋白、蓖麻毒蛋白 A-链; 辛纳毒蛋白、辛纳毒蛋白 A-链, IC_{50}增强活性比率分别为 3108, 15, 151, 45, 51 和 47, 7, 26, 12. **结论**: 首次发现 ATP 和 S-140 对Ⅱ型 RIP 及其 A-链具有活性增强作用. 克木毒蛋白和辛纳毒蛋白在对 ATP 和 S-140 的辅助需求方面有显著区别.