CHROMOSTIN, A NOVEL SPECIFIC INHIBITOR AGAINST REVERSE TRANSCRIPTASE

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We have been screening for enzyme inhibitors against avian myeloblastosis virus (AMV) reverse transcriptase in microbial metabolites, and discovered two low molecular weight inhibitors named retrostatin[1] and limocrocin[2]. Recently we found that a novel substance produced by an unidentified actinomycete, tentatively designated as Isolate H 1196 MY-6, showed highly specific inhibition against AMV reverse transcriptase with a much lower ID_{50} value when compared with those of retrostatin and rubistin. Since the active principle was a chromogenic protein, it was named chromostin. Taxonomy of the producing organism, and isolation and characterization of chromostin are described briefly in this paper.

The taxonomic studies of Isolate H 1196 MY-6 were carried out in accordance with the methods of the International Streptomyces Project (ISP)[3] and WAKSMAN[4]. The detection of the isomers of diaminopimelic acid (DAP) and carbohydrates were conducted by the methods of BECKER *et al.*[5] and LECHEVALIER[6], respectively. Isolate H 1196 MY-6 was well developed in all media tested. No fragmentation of the vegetative mycelium was observed. Neither sporangia nor flagellated spores were observed. The mature sporulated aerial mycelium was pale orange to light grayish brown in color, and it became hygroscopic within a week. The aerial mycelium bore long spore chains with 10~50 or more spores per chain arranged in spiral. Melanoid pigments were produced on peptone - yeast extract - iron agar. The spores were cylindrical in shape and measured 0.6~0.7 × 1.25~1.45 μm in size. The spore surface was smooth. Since the whole cell hydrolysate was found to contain *meso*-DAP, galactose and glucose, but not arabinose and xylose, the cell wall of Isolate H 1196 MY-6 was classified in type IIIC of LECHEVALIER and LECHEVALIER[7]. From these results, Isolate H 1196 MY-6 was considered to be an actinomycete distinguished from the genus *Streptomyces* in the family *Streptomycetaceae*.

Isolate H 1196 MY-6 was cultured to make an inoculum seed in a 500-ml Sakaguchi flask containing 100 ml of an inoculation medium composed of soluble starch 1.0% and yeast extract 0.2% (pH 7.0~7.2 before sterilization) at 27°C for 24 hours on a reciprocal shaker (amplitude 7 cm, 130 strokes/minute). Two ml of the seed culture was used to inoculate each 500-ml Sakaguchi flask containing 100 ml of a production medium composed of soluble starch 1.5%, glucose 1.0%, soybean meal 2.0%, Ebios (dried yeast distributed by Tanabe Pharmaceutical Co., Ltd.) 0.5%, NaCl 0.25% and $CaCO_3$ 0.3% (pH 7.6 before sterilization). The culture was conducted at 27°C for 96 hours on the same shaker.

The broth filtrate (1,400 ml) of Isolate H 1196 MY-6 was brought to 70% saturation with ammonium sulfate and the pH was adjusted to 7.0 by the addition of 1 N NH_4OH. The precipitate was collected by centrifugation, dissolved in distilled water, dialyzed against distilled water for 15 hours and lyophilized. The crude powder thus obtained (1,400 mg) was charged on a DEAE-cellulose column (20.0×3.2 cm, in diameter) which had been equilibrated with 10 mM Tris-HCl buffer (pH 8.0) and the column was eluted with a linear gradient concentration of NaCl (0~1.0 M) in the starting buffer. The active fractions were pooled, dialyzed against distilled water for 15 hours and lyophilized to give partially purified powder (282 mg) recovering 24% of the original activity. The partially purified powder (50 mg) was dissolved in 10 mM Tris-HCl buffer (pH 8.0, 1 ml) and charged on a Sephadex G-75 column (90.0×1.6 cm, in diameter) which was eluted with the same buffer. The active fractions were pooled, dialyzed against

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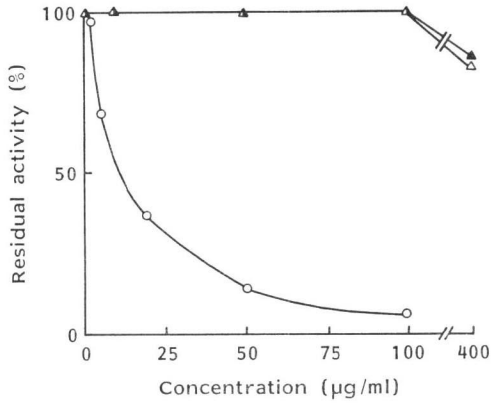
distilled water for 15 hours and lyophilized to give purified dark brown powder (34 mg). The purified powder was a chromoprotein showing a single band by electrophoresis on a cellulose acetate film and the molecular weight of chromostin was estimated to be 20,000 by gel filtration on Sephadex G-75. UV $\lambda_{max}^{H_2O}$ nm ($E_{1cm}^{1\%}$) 325 (64); $\lambda_{max}^{0.1N\ HCl}$ nm ($E_{1cm}^{1\%}$) 325 (60); $\lambda_{max}^{0.1N\ NaOH}$ nm ($E_{1cm}^{1\%}$) 275 (sh, 78) and 340 (sh, 50). Chromostin did not show any antimicrobial activity at the concentration of 1,600 μg/ml. No toxicity was observed in mice by ip injection of 500 mg/kg of chromostin. Inactivation of chromostin was not observed when it was allowed to stand at room temperature for 2 hours within a pH range of 2.0~12.0.

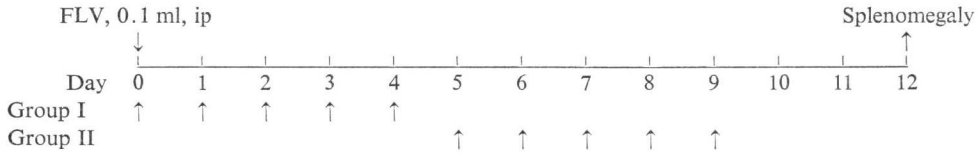
Assay methods for reverse transcriptase, DNA-directed DNA polymerase I of *Escherichia coli* and DNA-directed DNA polymerase α of calf thymus have been described in the other paper[2]. Chromostin preferentially inhibited AMV reverse transcriptase as seen in Fig. 1, showing 50% inhibition at 12 μg/ml, while DNA polymerase I and DNA polymerase α were not significantly affected at 400 μg/ml. As described in the other paper[2], ID_{50} values of adriamycin for AMV reverse transcriptase, DNA polymerase I and DNA polymerase α were 30 μg/ml, 20 μg/ml and less than 10 μg/ml, respectively. Because the decrease of AMV reverse transcriptase activity in the presence of chromostin was only compensated with increase in concentration of the enzyme, direct interaction between chromostin and the enzyme was postulated.

Fig. 1. Effects of chromostin on various DNA polymerases.
○ RNA-directed DNA polymerase of AMV.
△ DNA-directed DNA polymerase I of *E. coli*.
▲ DNA-directed DNA polymerase α of calf thymus.



We tested the effect of chromostin on Friend leukemia virus (FLV) infection of mice according

Table 1. Therapeutic effect of chromostin on Friend leukemia virus-infected mice.



Dose of chromostin (mg/mouse/day)		Weight of spleen (mg) mean±SD		% Inhibition*
		V^+C^+	Control V^-C^+	
Group I	1.0	432± 69	130±23	4.4
	0.5	396± 98	142±23	19.6
	0.25	454±107	132±15	−1.9
Group II	1.0	290±106	150±40	55.7
	0.5	386± 80	180±45	34.8
	0.25	410± 95	148±22	17.1
Control	V^+C^-	426± 73		
	V^-C^-	110± 20		

V^+ : Infected with FLV, V^-: not infected with virus.
C^+ : Chromostin-administered, C^-: chromostin-not administered.
* : $\%\ \text{Inhibition} = \left(1 - \frac{V^+C^+ - V^-C^+}{V^+C^- - V^-C^-}\right) \times 100$

to the method of NUMATA *et al.*[8]. FLV complex was prepared by transfection of molecularly cloned spleen focus forming virus (SFFV) DNA into NIH/3T3 cells and rescuing of the SFFV genome by helper virus[9,10]. Reconstituted virus thus obtained was inoculated into DBA/2 mice which were sacrificed 12 days after viral infection and a 10% homogenate of the enlarged spleens was used as a viral inoculum. Female DDD mice, 6-week old and weighing about 25 g, were injected with 0.1 ml of the viral inoculum. In group I, daily ip administration of chromostin was initiated on day 0 and continued for 5 days (see Table 1). In group II, the treatment was started on day 5 and lasted for 5 days. Control groups included mice infected with virus without following chromostin treatment, and uninfected mice with or without chromostin administration. Each experimental group consisted of 5 mice. Twelve days after the infection, all mice were killed and the spleens were taken out and weighed. The results are shown in Table 1. Chromostin inhibited splenomegaly dose-dependently in the case of administration on days 5~9, showing inhibition rates of 55.7%, 33.5% and 17.1% at doses of 1.0 mg/mouse/day, 0.5 mg/mouse/day and 0.25 mg/mouse/day, respectively. However, no distinctive inhibition was observed when chromostin was administered on days 0~4. A similar observation was reported by NUMATA *et al.*[8] using revistin as a chemotherapeutic agent. Though our results support the speculation by NUMATA *et al.*[8] that the effectiveness of the delayed treatment may be due to the more rapid viral replication during 5~9 days after the infection than at immediately after the infection, we can not exclude the possibility of indirect effect of chromostin such as stimulation of immune resistance.

Both revistin and chromostin are acidic proteins with the molecular weight of 20,000~30,000. The HCl-hydrolysate of chromostin gave all common amino acids, among which proline (13.9%), glutamate (12.5%) and valine (6.0%) were major constituents. On the other hand, revistin mainly consists of proline, glutamate and aspartate (NUMATA, personal communication). Although the producing organism of revistin was closely related to *Streptomyces filipinensis* (cell wall type I)[8], chromostin was produced by an actinomycete of cell wall type IIIC, belonging to the family *Streptomycetaceae*. Since the trial to isolate the chromophore of chromostin by the method of KOIDE *et al.*[11] was unsuccessful, the roles of the chromophore and protein component still remain to be elucidated.

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