Communications to the editor

ISOLATION OF A NON-PROTEIN COMPONENT AND A PROTEIN COMPONENT FROM NEOCARZINOSTATIN (NCS) AND THEIR BIOLOGICAL ACTIVITIES

Sir:

An antitumor antibiotic of protein nature[1], neocarzinostatin (NCS), was isolated in 1965 from culture filtrates of *Streptomyces carzinostaticus* var. F-41 by ISHIDA *et al.*[2] It has been reported that NCS inhibits DNA synthesis[3] and cell division[4], and it causes DNA strand breakage[5,6] and induces DNA repair synthesis[7,8]. Clinically, NCS has been used for the treatment of patients with acute leukemia[9], gastric cancer[10], and pancreas cancer[11].

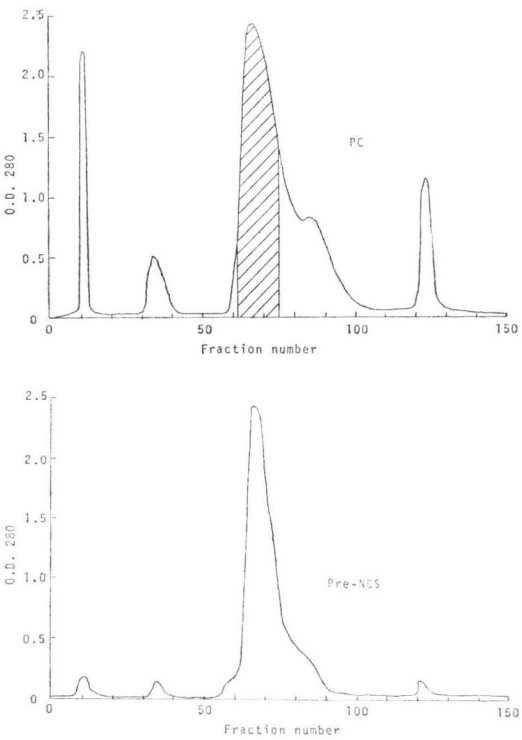
Pre-NCS, an acidic protein isolated in 1974 from culture filtrates of the same *Streptomyces*[12] is very similar to NCS in amino acid composition but has no biological activities. This protein can also be derived from NCS by ultraviolet (UV) irradiation[13] or by dialysis of an aqueous solution of NCS under acidic conditions[14].

Recently, NAPIER *et al.*[15] reported that NCS molecule contained a chromophore in addition to a protein moiety, based on the results of UV absorption, circular dichroism, and magnetic circular dichroism spectra of NCS, before and after methanol treatment. ISEKI *et al.*[13] suggested the presence of a non-protein component (NPC) in NCS molecule by comparing the biological activities of Pre-NCS and UV irradiated NCS. This paper deals with the isolation, purification, and biological activities of NPC and protein component (PC) obtained from NCS.

Purified NCS powder (clinical grade 1,500 units/mg), produced in Kayaku Antibiotics Research Laboratory, was used. NPC and PC were separated as follows: A suspension of NCS powder (30 g) in 600 ml 1 N HCl H_2O - MeOH (1: 5) was stirred for 2 hours at 4°C in a dark room, and then centrifuged at 9,000 rpm for 20 minutes. The precipitate was dried to a brownish powder (26 g) and was used for the the purification of PC, whereas the supernatant, for that of NPC. Purified PC (135 mg) was obtained from brownish powder (1 g) by gel filtration on a Sephadex G-25 and a carboxymethyl (CM)-cellulose column chromatography, by collecting main fractions showing absorption at 280 nm but not at 300~350 nm (Fig. 1). The supernatant was concentrated *in vacuo* to dryness. The residue, redissolved in 100 ml of 1 N HCl H_2O - MeOH (1: 9), was chromatographed on a Sephadex LH-20 column (31 mmϕ × 138 cm). NPC was eluted with 1 N HCl H_2O - MeOH (1: 9). The inhibition of growth

Fig. 1. CM-cellulose chromatography of PC and Pre-NCS.

Five ml samples of crude PC (600 mg) and Pre-NCS (300 mg) were applied on a CM-cellulose column (20 mmø × 40 cm) and eluted successively with 0.1 M acetic acid (200 ml), 0.1 M acetate buffer pH 3.3 (900 ml), and 0.1 M acetate buffer pH 3.7 (400 ml). Each fractions contained 10 ml eluate and measured for the UV absorption at 280 nm. Fractions 62~75 were combined and used as PC (cross-hatched area).



of *Sarcina lutea* was used for the detection of NPC. Active fractions were combined and evaporated to dryness to yield 263 mg of the brownish crystalline NPC. All purification procedures were done in a dark room.

Isoelectric focusing on polyacrylamide gel was carried out for the identification of PC according to the method of KARLSSON *et al.*[16] Acute toxicity of both NPC and PC were examined in *ddY* mice and antitumor effect on YOSHIDA sarcoma in Donryu rats by ip-ip method[17].

The isolated NPC decomposed at 125°C and was soluble in methanol but not in water. NPC (1 mg/ml) was negative in ninhydrin and biuret reactions, but it decolorized potassium permanganate solution and underwent a coupling reaction with diazonium salts with the formation of red-brownish color. This and the UV absorption spectra (Fig. 2) suggest the presence of an aromatic moiety. Furthermore, NPC exhibited an optical rotation of $[\alpha]_D^{20}$ $-171°$ in 1% methanol solution, and a band 1780 cm^{-1} in IR spectrum (KBr) (Fig. 3) suggested the presence of lactam or cyclic ketone. The molecular weight of NPC was estimated to be between 500 and 1,000 by a Sephadex LH-20 column chromatography.

On the other hand, purified PC was insoluble in methanol and was eluted in the same fraction as Pre-NCS by a CM-cellulose column chromatography (Fig. 1). The purified PC showed a

Fig. 2. UV absorption spectra of non-protein component (NPC), protein component (PC), and neocarzinostatin (NCS).

The spectra were measured by Hitachi Spectrophotometer Model 624 to 1 cm quartz cell. A: NPC, 50 mcg/ml in methanol. B: NCS, 500 mcg/ml in H_2O. C: PC, 500 mcg/ml in H_2O.

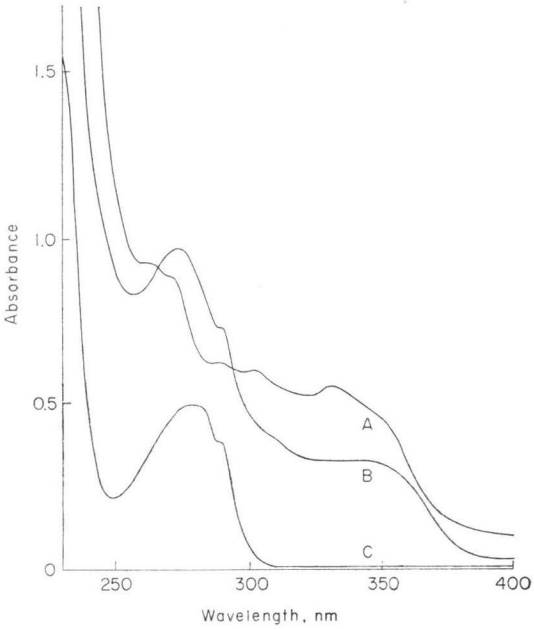


Fig. 3. IR spectrum (KBr) of non-protein component (NPC).
The spectrum was measured by a JASCO IRA-1 spectrometer.

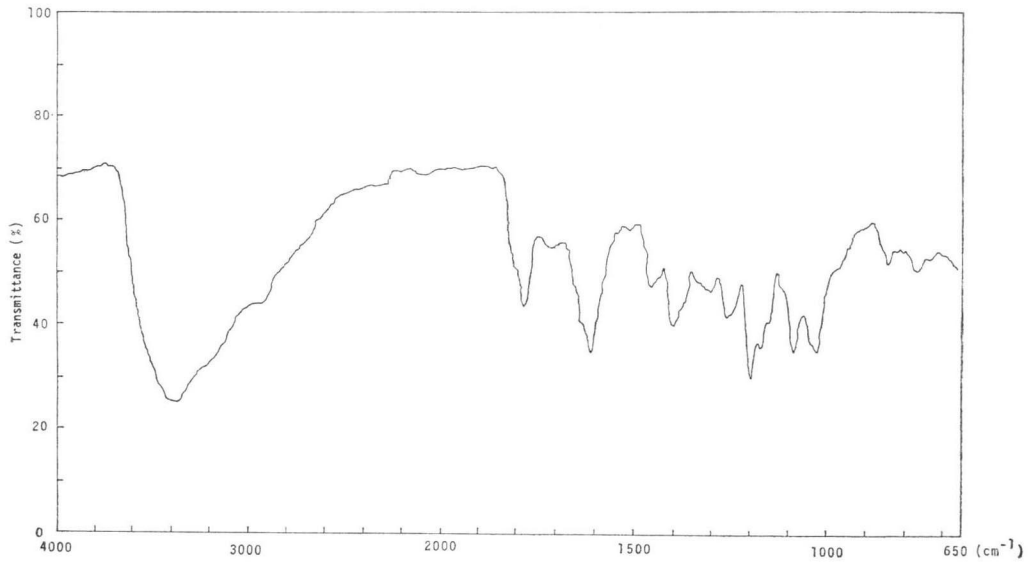


Table 1. Comparison of the antimicrobial spectrum of NPC, PC, NPC in the presence of PC, and NCS.

Test microorganisms	Minimum inhibitory concentration (mcg/ml)			
	NPC	PC	NPC*+PC	NCS
Staphylococcus aureus FDA209P	50	>100	1.56	6.2
Staphylococcus epidermidis IFO3726	25	>100	0.4	6.2
Sarcina lutea ATCC9341	12.5	>100	0.1	3.1
Streptococcus haemolyticus	25	>100	0.2	6.2
Streptococcus faecalis IFO12580	25	>100	0.4	6.2
Bacillus subtilis PCI219	25	>100	0.8	12.5
Bacillus cereus IFO3001	>100	>100	0.8	12.5
Bacillus licheniformis ATCC14580	>100	>100	3.1	12.5
Proteus vulgaris OX19	>100	>100	>100	>100
Proteus morganii IFO3848	>100	>100	>100	>100
Proteus rettgeri	>100	>100	>100	>100
Salmonella typhimurium	>100	>100	>100	>100
Salmonella paratyphi A1015	>100	>100	>100	>100
Salmonella typhi V1901	>100	>100	>100	>100
Shigella flexneri 2a	>100	>100	>100	>100
Shigella sonnei	>100	>100	>100	>100
Escherichia coli NIHJ	>100	>100	>100	>100
Pseudomonas aeruginosa GN83	>100	>100	>100	>100
Serratia marcescens IAM1022	>100	>100	>100	>100
Klebsiella pneumoniae GN350	>100	>100	>100	>100
Citrobactor freundii Ball07	>100	>100	>100	>100
Xanthomonas oryzae IAM1657	>100	>100	>100	>100
Candida albicans YU1200	>100	>100	>100	>100

* Minimum inhibitory concentration of NPC on plate contained 10 mcg per ml of PC.

single band on polyacrylamide gel isoelectric focusing and revealed the same mobility as that of Pre-NCS. Additionally, UV spectrum of PC was very similar to Pre-NCS (data not shown).

Fig. 2 shows the UV absorption spectra of NPC, PC, and NCS. The spectrum of PC was different from that of NCS in the disappearance of 310~350 nm absorption, whereas λ_{max} at 280 nm was found as in the case of Pre-NCS[13]. NPC in methanol showed UV maxima at 265, 270, 290, 305 and 330 nm. However, no characteristic absorption at 280 nm of PC and at 274 nm of NCS was detected in the spectrum of NPC.

The LD_{50} values for NPC by i.v. and i.p. injection in mice were 1.8 mg/kg and more than 10 mg/kg, respectively, while those of PC after i.v. and i.p. administrations were more than 500 mg/kg.

The antimicrobial activities of the isolated components NPC and PC, were compared with those of NCS[18]. As shown in Table 1, NPC alone showed significant growth inhibition at the range of 12.5~50 mcg/ml against *S. lutea*, *Staphylococcus*, and *Bacillus subtilis*, following almost the same spectra of NCS, which ranged from 3.1~12.5 mcg/ml. In other words, NCS is almost 4 times more active than NPC, however both of them are inactive against Gram-negative bacteria. PC did not reveal bacteriostatic activities against the Gram-positive or negative bacteria so far tested. However, when the antimicrobial activities of NPC were examined in the presence of PC (which had been incorporated into agar plate), the minimum inhibitory concentration, as low as 0.1 mcg/ml, was obtained against *S. lutea*, indicating 4 times activity of that of NCS.

Table 2 shows preliminary studies of the antitumor activity of NPC, PC and the mixture in rats bearing YOSHIDA sarcoma. When a single dose was given i.p. 72 hours after implantation, NPC alone showed definite cytostatic activity on day 5 at 0.1 mg/kg dose. PC alone was ineffective under the same schedule, even at 10 mg/kg dose. When the mixture of NPC-PC

Table 2. Synergic action of NPC and PC against YOSHIDA sarcoma.

	MTD* mg/kg	MED** mg/kg
NPC	>10.0	0.1
PC	>100.0	>10.0
NPC+PC (1:10)	0.5	0.005
NCS	2.5	0.05

* MTD: maximum tolerated dose when given i.p.
** MED: minimum effective dose when given i.p.

(1:10) was examined in the same way, it was effective at a dose as low as 0.005 mg/kg (on NPC base), while the original NCS revealed the same activity at 0.05 mg/kg. However, when the chemotherapeutic index was compared between the mixture and NCS (Table 2), the index of 100 obtained with the mixture was not significantly different from that of 50 obtained with NCS.

In summary, NPC alone was found to have both antimicrobial and anti-YOSHIDA sarcoma activities but PC alone had no activities. However, the biological activities of NPC were greatly enhanced by the presence of PC, although the critical concentration ratio of the two components showing the highest activity was not examined. More important is the fact that NPC alone showed the same antimicrobial spectrum and anti-YOSHIDA sarcoma activity as did NCS. Furthermore, NPC alone inhibited DNA synthesis in sensitive cells and induced DNA strand breakage but PC did not (data not shown, in preparation). Thus it is suggested that NPC may be responsible for the biological activities of NCS, although the exact role of PC has not yet been elucidated in both biological and chemical terms.

The isolation of NPC from the NCS molecule is reminiscent of the isolation of Fraction B from the carzinostatin complex[19], which had been obtained from culture filtrates of *Streptomyces carzinostaticus*. A review of the literature revealed that the antitumor protein, auromomycin, inhibitory to the growth of both Gram-positive and -negative bacteria, was converted to macromomycin after passing through Amberlite XAD-7 chromatography[20] with the loss of UV absorption at 350~360 nm. This information suggests that a chromophore in auromomycin might be related to NPC reported here, however more exact comparisons of the two chromophoric components are left for future studies. The investigation to determine the structure of NPC derived from NCS is now in progress.

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