Communications to the Editors

TRYPTIC DIGESTION OF AN ANTITUMOR PROTEIN : NEOCARZINOSTATIN

Sir :

Neocarzinostatin, produced by a strain of *Streptomyces*, is an acidic protein (M. wt. 9,000) which exhibits antitumor properties in animals[1] and inhibits DNA synthesis or induces DNA-degradation in bacteria[2,3]. The amino acid composition, N- and C-terminal amino acids, and conformational study have been reported from our laboratory[4,5,6]. It has been reported that this antitumor protein retains its biologic activity after treatment with proteolytic enzymes such as trypsin, chymotrypsin, papain, Nagarse and Pronase under optimum conditions[4]. Inhibition of proteolytic enzymes did not occur[4]. Since neocarzinostatin is composed of L-type amino acids[4,7], it is suggested that active peptides may be formed by the action of proteolytic enzymes.

Neocarzinostatin was purified according to the method of MAEDA *et al.*[4] with slight modifications. The material was purified by repeated rechromatography on CM-cellulose (three times) and gel filtration (Sephadex

Fig. 1. Biological activity of tryptic digest of neocarzinostatin.

Percent of DNA degradation was measured for the trypsin-treated neocarzinostatin in *Sarcina lutea* after 60 min. drug treatment at 37°C. Trypsin 0.6 mg was added to the neocarzinostatin (25 mg) solution in 1 ml tris-HCl buffer at pH 8.2 with an appropriate amount of $CaCl_2$. The reaction was conducted for 48 hours at 27°C, the pH after the treatment was about 8.0. Then the reaction mixture was chromatographed on Sephadex G-25 (1.3×42.5 cm). Elution was made with 0.1 M acetate buffer pH 4.4 and each tube contained 3 ml of the fraction. For the assay of DNA degradation, 0.1 ml of the fraction was taken.

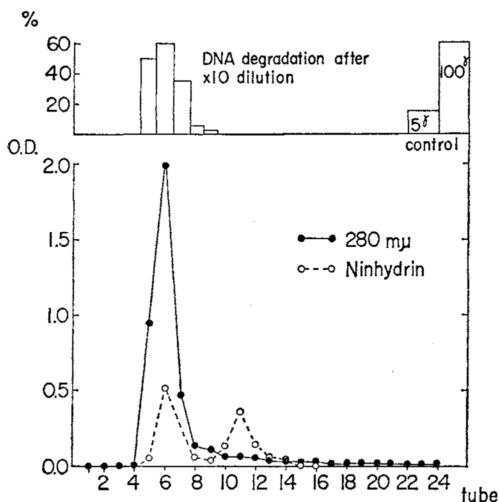
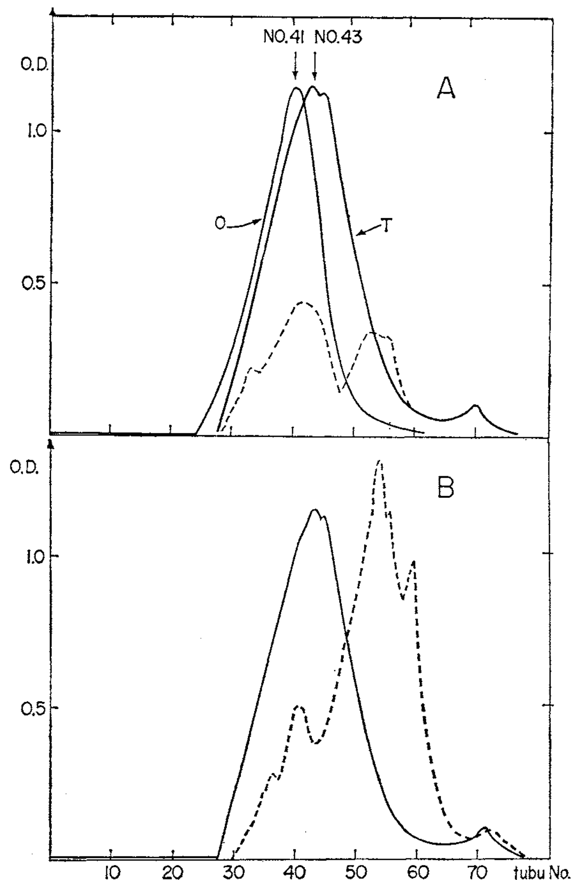


Fig. 2. Gel filtration of trypsin-treated neocarzinostatin by Sephadex G-50.

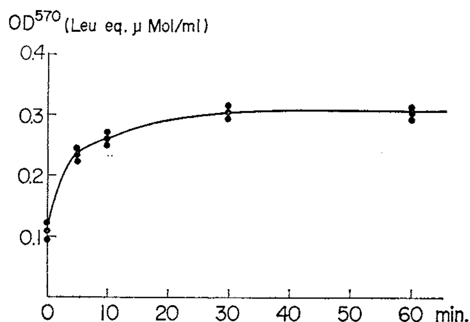
Fig. 2-A shows chromatographic patterns of original and trypsin-treated neocarzinostatin measured by absorption at 280 mμ. The condition of the treatments are described in the text. T and O indicate treated and original. Ninhydrin assay (O. D. 570 mμ) was paralleled (dashed line) to O. D. measurement at 280 mμ for the trypsin-treated material. In Fig. 2-B, trypsin treatment was made after heat denaturation of neocarzinostatin (90°C, for 20 minutes).



G-50). The material was pure as judged by ultracentrifugation, polyacrylamide gel electrophoresis, polyacrylamide disc electrophoresis or N-terminal amino acid analysis. Crystalline trypsin was purchased from Boehrlinger & Soehne GmbH (Mannheim, Germany) and treated with TPCK (Tosyl-L-phenylalanine chloromethyl ketone) according to SCHOELLMAN and SHAW[8] to eliminate contaminating chymotrypsin. The ninhydrin method was similar to that of YEMM and COCKING[9]. The degree of DNA degradation was measured as described previously[2]. The trypsin treatment was made, unless otherwise noted, at pH 8.3 in 0.1 M Tris-HCl buffer with an appropriate amount of Ca^{++} at 37°C. The ratio of the enzyme to substrate was 1/30 to 1/100.

Fig. 3. Time course study of increment of amino groups after trypsin treatment of neocarzinostatin.

Optical density read at 570 mμ after the ninhydrin reaction and appropriate dilution.



In Fig. 1, the activity of trypsin treated neocarzinostatin after gel filtration (Sephadex G-25) is measured by the extent of DNA degradation in *Sarcina lutea*. Two ninhydrin positive peaks appeared, one at higher (>2.000) and another at lower molecular weight region. The latter fraction showed no significant biologic activity by this test. The peak containing the biologic activity seems to be a large peptide with 280 mμ absorption. In Fig. 2-A, the results of gel filtration of original (O) and trypsin treated (T) neocarzinostatin with Sephadex G-50 (1.5×110 cm) are shown. The eluates were measured by ultraviolet absorption at 280 mμ (solid line) and ninhydrin reaction (dash line). In Fig. 2-B, gel filtration of neocarzinostatin which had been denatured and then treated with trypsin is shown.

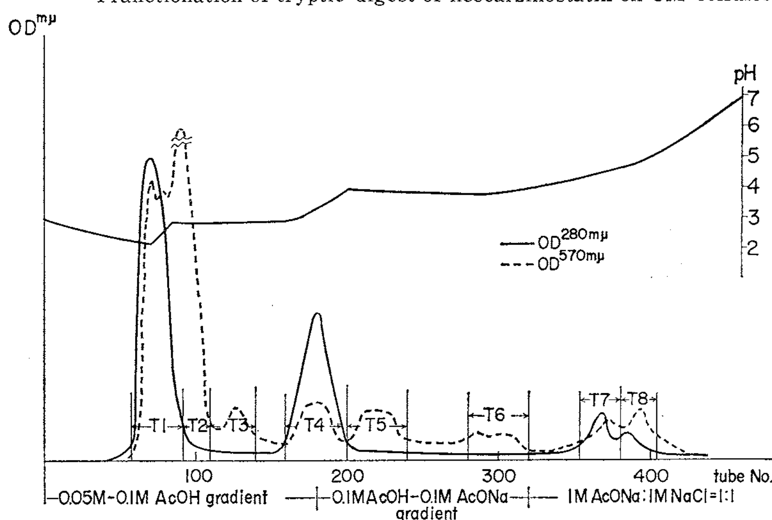
The results shown in Fig. 2 A and B indicate that tryptic digestion decreases the molecular weight to a slight extent (compare (T) and (O)). The trypsin treatment results in formation of a smaller ninhydrin-positive peptide. This reaction is enhanced when the protein is denatured by heat (90°C for 20 minutes) prior to the tryptic digestion (Fig. 2-B).

Fig. 4. CM-cellulose chromatography of trypsin-treated neocarzinostatin

The condition of the enzyme treatment is described in the text.

High ninhydrin peak corresponds to urea added for the trypsin digestion.

Franctionation of tryptic-digest of neocarzinostatin on CM-cellulose



The increase of free amino groups upon tryptic digestions was measured by ninhydrin reaction (Fig. 3), where a 1 : 50 ratio of trypsin to substrate was used. An aliquot equivalent to 0.1 μM of neocarzinostatin taken at an appropriate reaction time, was brought to pH 4.5 with 1 M acetic acid, boiled for 15 minutes to stop the proteolysis, and frozen until ninhydrin assay. The results show an increase of amino groups to almost three times that of the original.

Neocarzinostatin (native, 50 mg) treated with trypsin in the presence of 2 M urea for 20 hours was fractionated on CM-cellulose with the results shown in Fig. 4. The elution was initially made with 0.05 M acetic acid, followed by 0.05 M acetic acid - 0.1 M acetate and 0.1 M acetate - 0.1 M Na acetate in linear pH gradient fashion. The elution was completed with a mixture of 1 M Na acetate and 1 M NaCl. Potent activity was found in peaks T1, T4 and T7, which inhibited the growth of *Sarcina lutea* and HeLa cells.

However, a complete separation of the major peptides (there are four peptides as will be discussed below) could not be attained. Since the protein possesses two S-S bridges in the molecule, a complete digestion of the molecule into simple peptide was not expected, as the peptides may be inter-linked by S-S bridge(s). This must be true because two N-terminal amino acids, alanine and valine, were found in T1 fraction. Probably T1 has two peptides, namely alanyl and valyl peptide inter-linked by S-S bridge(s).

The above results afford the following conclusions:

(1) Neocarzinostatin retained biological activity even after tryptic digestion, where a small peptide was cleaved from the molecule.

(2) Trypsin hydrolysis of the protein yields a peptide of lower molecular weight, about 7,000 (Fig. 2), and heat denaturation enhances the formation of this lower molecular-weight peptide.

(3) The appearance of amino groups with time upon tryptic digestion of the protein indicates that tryptic hydrolysis is complete after 30 minutes at 27°C (Fig. 3). The amino group content increases to almost three times that of native neocarzinostatin. There are three sites (1 lys and 2 arg) for the tryptic cleavage which could result in the formation of four peptide chains. The original N-terminal amino group plus three newly formed N-terminal amino groups, together with one original lysyl ε-amino should yield theoretically 4.78 mole leucine equivalent value assuming ε-amino of lysine corresponds to 78 % of leucine equivalency[10], that the theoretical value for neocarzinostatin is 1.78 mole leucine equivalent. Dividing 4.78 by 1.78, one obtains a theoretical increase in amino groups of 2.70 fold (leucine equivalents). Experimentally the increase was 2.73 fold. These facts indicate that complete hydrolysis has been attained, although disulfide bridges have made the peptide separation difficult.

(4) Fractionation of these tryptic peptides by CM-cellulose indicated the presence of active peptides smaller than the original protein. Characterization of the peptides is under way.

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