A SPECIFIC INHIBITOR FOR TYROSINE PROTEIN KINASE FROM *PSEUDOMONAS*

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An effective antitumor substance is one of the most exciting targets for researchers in the chemotherapeutic field. Recently, it was found that oncogenes transform the normal cells and cause various types of tumors in human as well as in animals[1]. Some of them, such as *src*, *fps*, *fes*, *yes* and *ros*, code for tyrosine-specific protein kinase[2], which may phosphorylate target proteins and trigger cellular transformation. According to this theory, it is a hopeful way for overcoming the cancer to find a specific inhibitor against the tyrosine-specific protein kinase. In this line of screening program, we isolated genistein from fermentation broth of *Pseudomonas* sp. This report describes the first isolation of genistein from *Pseudomonas*, which is an inhibitor against tyrosine-specific protein kinase and may be a promising antitumor substance.

The microorganism used for the isolation of genistein was isolated from a soil sample. Based on the cultural and physiological properties, strain YO-0170J was similar to *Pseudomonas stutzei* and *P. mendocina*. However, in contrast to *P. stutzei*, strain YO-0170J could not hydrolyze starch nor denitrify nitrate and nitrite. In addition, utilization pattern of carbon sources was different between the two. On the other hand, strain YO-0170J was quite similar to *P. mendocina* except that the latter formed yellowish soluble pigment.

Protein kinase activity of epidermal growth factor (EGF) receptor was determined in a final volume of 50 μl containing 20 mM HEPES-NaOH, pH 7.2, 10 mM $MgCl_2$, 3 mM $MnCl_2$, 1 mM dithiothreitol, 20 μM [γ-^{32}P]ATP (4 mCi/μmol), 100 ng/ml mouse EGF (Collaborative Research), A431 cell membrane (5 μg protein) and the kinase inhibitor. After 5 minutes at 0°C, the reactions were terminated by the addition of LAEMMLI's sample buffer[3] and by boiling for 2 minutes. The samples were analyzed by SDS polyacrylamide gel electrophoresis and autoradiography.

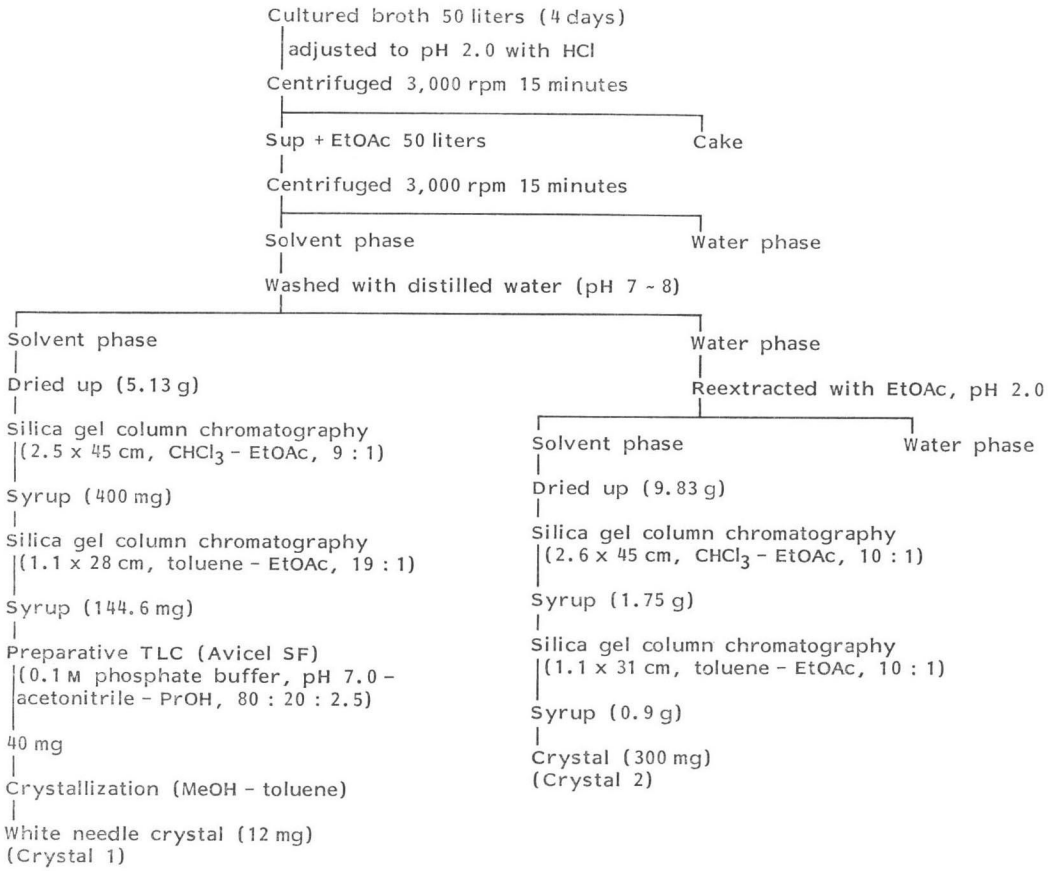
The *src* kinase activity was assayed as follows: Rous sarcoma virus (RSV)-transformed 3Y1 cells were lyzed with solubilizing buffer (1% Nonidet P-40, 0.1% sodium deoxycholate, 0.15 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 50 mM Tris-HCl, pH 7.4) and clarified by centrifugation at 100,000$\times g$ for 20 minutes. The supernatant was immuno-precipitated with antisera obtained from rabbits bearing tumors induced by RSV (TBR sera) and protein A-Sepharose 4B. The kinase activity of the immuno-precipitates was assayed by incubating with [γ-^{32}P]ATP (4 mCi/μmol) in a final volume of 50 μl containing 20 mM HEPES-NaOH, pH 7.2, 5 mM $MgCl_2$, 1 mM dithiothreitol and the kinase inhibitor. The reactions were continued for 5 minutes at 25°C and stopped by the addition of LAEMMLI's sample buffer. The products of the reactions were resolved by SDS-polyacrylamide gel electrophoresis and autoradiography. The inhibitory activity was calculated from the remaining protein kinase activity.

Human epidermoid carcinoma A431 cells and rat 3Y1 cells transformed by RSV were grown in DULBECCO's modified EAGLE's medium supplemented with 7% fetal calf serum.

A loopful of cells of the strain YO-0170J on an agar slant were inoculated into a 500-ml Erlenmeyer flask containing 60 ml of a medium composed of glucose 3%, dextrin 3%, SIII Meat (Ajinomoto Co.) 1.5%, Fermamedia (Buckeye Cellulose Corp.) 1.5%, K_2HPO_4 0.06%, KH_2PO_4 0.025% and $CoCl_2$ 0.0004%. The pH was adjusted to 7.0 before sterilization. The flasks were incubated on a rotary shaker at 28°C for 3 days. The culture was transferred to new flasks containing 60 ml of the same medium at 3% and the fermentation was continued at 28°C for 4 days with shaking.

The purification procedure is outlined in Fig. 1. Crystal 1 was identified as genistein by high resolution mass spectroscopy (molecular weight of 270.05243) and ^{1}H NMR and ^{13}C NMR spectroscopy. The IR and UV absorption spectra confirmed this. This showed ID_{50} for the tyrosine-specific protein kinase activity of EGF re-

Fig. 1. Purification procedure of genistein and *p*-hydroxybenzoic acid from *Pseudomonas* sp. YO-0170J.



ceptor (A431 cell membrane) at 0.7 μg/ml, and that of RSV-transformed 3Y1 cells at 8.0 μg/ml. However, it showed no inhibitory activity against cAMP-dependent protein kinases at 100 μg/ml. On the other hand, the water phase was reextracted with ethyl acetate (50 liters) at pH 2.0 and the solvent was evaporated to dryness to give 9.83 g of brownish powder. After two cycles of silica gel column chromatography as shown in Fig. 1 and recrystallization from $CHCl_3$, 300 mg of white crystals (Crystal 2) were obtained. Crystal 2 was identified as *p*-hydroxybenzoic acid by using mass and ^1H NMR spectroscopy and thin-layer chromatography. ID_{50} for the tyrosine-specific protein kinase activity of EGF receptor (A431 cell membrane) was 10 μg/ml.

An isoflavone compound, genistein, was isolated from subterranean clover (*Trifolium subterraneum* L.) as a main compound responsible for its oestrogenic activity[4]. Recently, it was isolated from the culture filtrate of *Streptomyces xanthophaeus* as an inhibitor against β-galactosidase[5]. However, no report was published on the isolation of genistein from *Pseudomonas* sp. In addition, as far as we know, this is the first paper describing the isolation of an isoflavone compound as a specific inhibitor against tyrosine-specific protein kinases. It did not show any toxic effect on mouse C57BL/6 at 500 mg/kg (ip). Therefore, it may become a valuable anti-tumor substance as well as a useful reagent for the study of tyrosine-specific protein kinases in cells.

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