POLY-(L)-MALIC ACID; A NEW PROTEASE INHIBITOR FROM PENICILLIUM CYCLOPIUM

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Occurrence of substances showing inhibitory action on trypsin and microbial proteinases has been demonstrated in a variety of biological materials including beans, potato, egg white, and blood. However, little has been known about inhibitors produced by microorganisms, except for the report by Hoeyen and Skulberg (1962) of the existence of trypsin inhibitor in the culture filtrate of Clostridium botulinum. Recently, we have found a protease inhibitor in the aqueous extract of wheat bran culture of Penicillium cyclopium (K.Shimada and K.Matsushima,1967), and the inhibitor has been purified and partially characterized (K.Shimada and K.Matsushima,1969). It is an acidic substance of high molecular weight, and is lacking nitrogen. These characteristics of the inhibitor have attracted our special interest since naturally occurring protease inhibitors so far known are protein.

This paper presents the study on the identification of the inhibitor. On heating with 0.2 N hydrochloric acid at 100°C for 3 hr, the inhibitor gave malic acid, as identified by paper chromatography and by elution analysis on a column of silica gel, in almost perfect yield (98 %). The resulting malic acid was of the L-configuration, the rotation agreeing closely to that of the au-

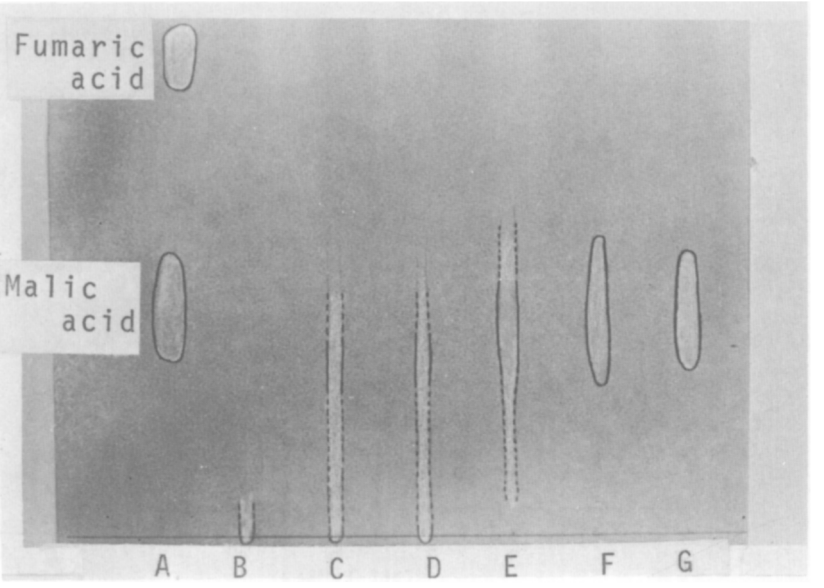
thentic L-malic acid. The inhibitor was thus found to be a kind of polyacid consisting of L-malic acid.

MATERIALS AND METHODS

The inhibitor was obtained from the aqueous extract of wheat bran culture of Penicillium cyclopium according to the procedure described before (K.Shimada and K.Matsushima,1969). This preparation was ascertained to be of homogeneous nature by sedimentation analysis. The hydrolysis of the inhibitor was performed in a sealed tube with 0.2 N hydrochloric acid at 100°C at an inhibitor concentration of 5 mg per ml. The hydrolyzates were dried under vacuum in a desiccator which contained both phosphorous pentoxide and sodium hydroxide. One way ascending chromatography on Toyo Roshi No. 50 paper was accomplished using n-butanol-formic acid-water (4 : 1.5 : 1), n-butanol-acetic acid-water (2 : 1 : 1), and ethyl acetate-formic acid-water (70 : 20 : 10) as solvents. Acids were detected on paper with bromophenol blue spray reagent. Elution analysis on a column of silica gel (prepared from Mallinckrodt's silicic acid specially prepared for chromatographic analysis) was carried out by the method of Bullen et al. (1952) with a series of n-butanol-chloroform solvents (100 ml of 5 %, 135 ml of 15 %, 100 ml of 25 %, and 300 ml of 35 % n-butanol-chloroform). Fractions of 3.3 ml were collected and were titrated by the addition of 0.01 N sodium hydroxide in the presence of phenol red indicator. Rotation was determined with a Shimazu polarimeter according to the method of Krebs and Eggleston (1943). Determination of malic acid was carried out by 2,7-naphthalenediol method of Goodban and Stark (1957).

RESULTS AND DISCUSSION

Since the inhibitor has been shown to be an acidic macromolecular substance (K.Shimada and K.Matsushima, 1969), we, on an



A; markers, B; untreated inhibitor, C; boiled in water for 30 min, D - G; hydrolyzed with 0.2 N HCl at 100°C for 0.5, 1.5, 3, and 5 hr, respectively.

Fig. 1. Paperchromatogram of hydrolysates of the inhibitor. Solvent; n-butanol-formic acid-water (4 : 1.5 : 1), upper phase. Locator; bromphenol blue.

assumption that it might be a polymerized form of an organic acid, checked the occurrence of organic acids in the hydrolyzate of the inhibitor preparation. Paper chromatographic analysis with the above solvent systems revealed that the hydrolysis of the inhibitor preparation with 0.2 N hydrochloric acid for more than 3 hr gave rise to a single spot on a paper. The Rf value in each solvent system agreed closely with that obtained with authentic L-malic acid. In Fig. 1 is shown a typical result. The elution profile from a column of silica gel also confirmed the above finding. In agreement with the result with authentic L-malic acid,

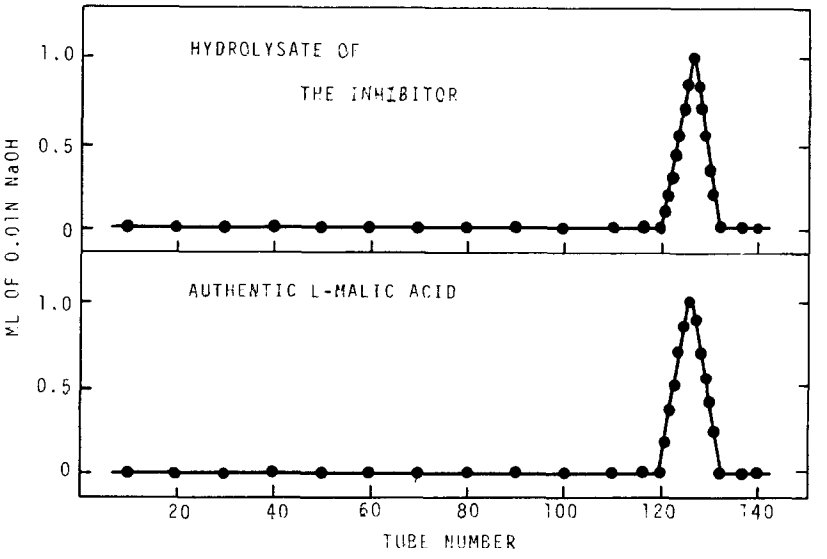


Fig. 2. Chromatograms of hydrolysate of the inhibitor and L-malic acid on silica gel column.

the hydrolyzate of the inhibitor localized in a single symmetric peak in fraction numbers between 120 and 132 (see Fig. 2).

Quantitative analysis indicated that 100 mg of the inhibitor preparation gave rise to 112 mg of malic acid by hydrolysis. The yield corresponded to 97.6 % of the calculated value on a basis of the inhibitor molecular weight of 5000 (K.Shimada and K.Matsushima, 1969). It seemed, therefore, most reasonable to assume that the inhibitor is composed solely of malic acid.

The rotation of the malic acid in the hydrolyzate of the inhibitor agreed closely with that of authentic L-malic acid; $[\alpha]_D^{20}$ of the sample and of authentic L-malic acid at a concentration of 0.125 mg per ml were +1381° and +1404°, respectively.

Thus, it is possible to conclude from the results given above that the inhibitor is poly-(L)-malic acid. The presence of ester linkages in the inhibitor molecule was presumed by the absorption peaks in the infrared spectrum at 1740 cm^{-1}, 1180 cm^{-1},

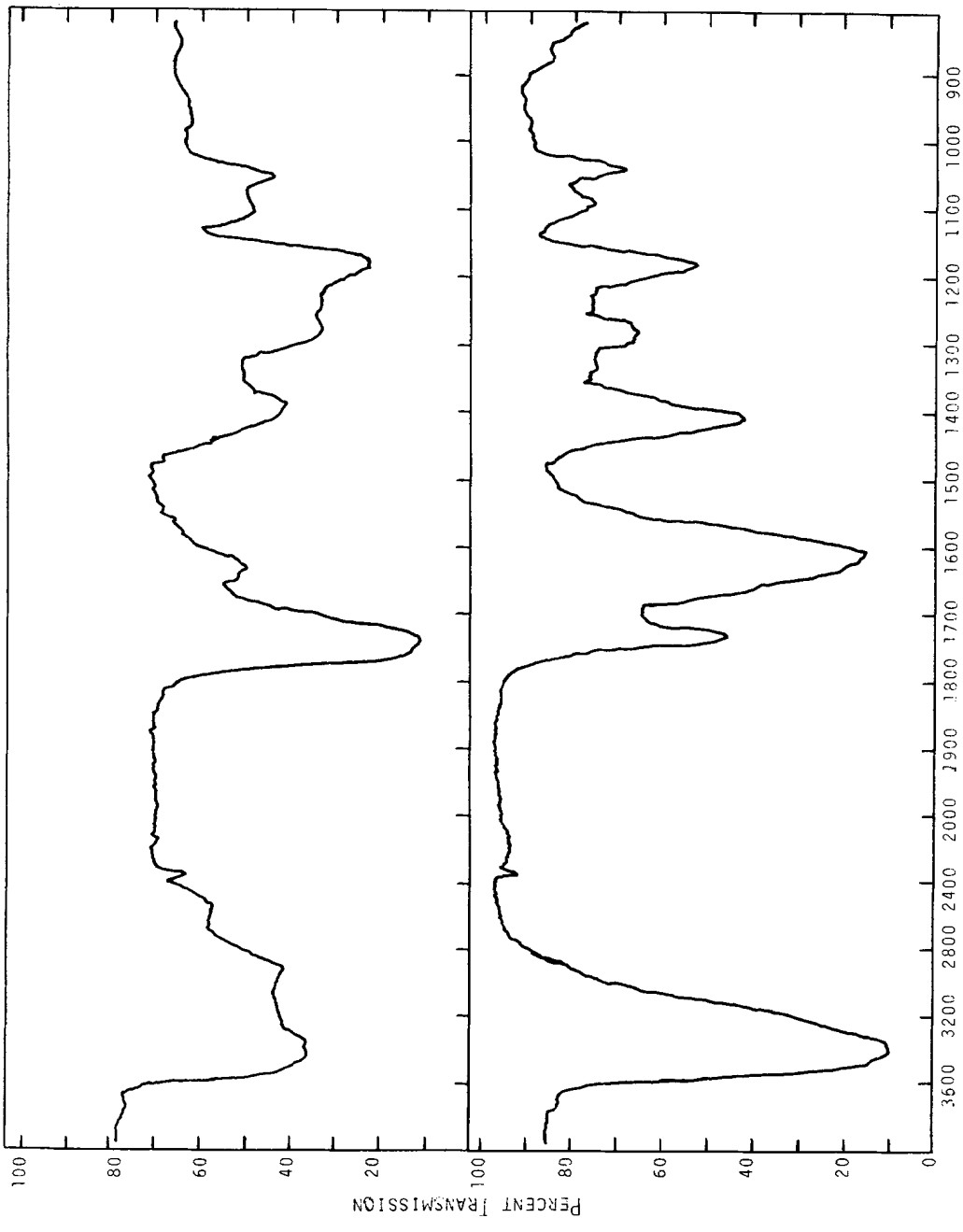


Fig. 3. Infrared absorption spectra of the inhibitor and its sodium salt (Kbr tablet). Above; the inhibitor, bottom; sodium salt of the inhibitor.

and 1050 cm^{-1}, and that of carboxylate anion at 1600 cm^{-1} with sodium salt of the inhibitor (see Fig. 3). The content of carboxyl group of the inhibitor was measured as 8.8 mmoles per gram of the inhibitor by the acid titration method. These results lead us to the assumption that one carboxyl group of malic acid combines with the hydroxyl group of the neighboring malic acid to form ester linkage, thus each malic acid residue possesing one free carboxyl group.

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