the surface of the protoplasts. The acid phosphatase inside the protoplast increased from 0.3 to 0.8 units/10^6 cells in 2 h and then the level dropped to 0.2 units at 3 h. The alkaline phosphatase inside the protoplast remained at 3.6 units/10^6 cells throughout the course of the incubation and none was found in the medium. The fact that no alkaline phosphatase was present in the medium confirms the view that the secreted acid phosphatase was not released by lysis of the protoplasts. For comparison the invertase synthesized and secreted into the medium is shown (Fig. 1). As invertase[6,7], the acid phosphatase of yeast is external to the cell membrane, and in the absence of cell wall newly synthesized enzyme is secreted into the medium.

This work was supported in part by grants from the National Science Foundation (G 9863) and U.S. Public Health Service (E 4572).

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Received September 3rd, 1962

SC 11026

Separation of a Tribolium-protease inhibitor from soybeans on a calcium phosphate column

The preparation of a protein fraction (C_1) from soybean meal which inhibits growth and proteolytic activity *in vitro* of *Tribolium confusum* larvae and trypsin has been reported by LIPKE *et al.*[1]. It has also been found that C_1 inhibits α-chymotrypsin[2] and possesses a strong amylase activity[3]. The present study comprises an attempt to separate the Tribolium inhibitor of C_1 from the accompanying trypsin inhibitor and soybean amylase.

Proteolytic and inhibitory activity was determined by the casein digestion method[4]. Amylase activity was determined by the method of NOELTING AND BERNFELD[5] using the modified 3,5-dinitrosalicylic acid reagent. Larval enzyme solutions were prepared by dissecting out midguts of last-instar larvae. The midguts were then homogenized and centrifuged as described by BIRK AND APPLEBAUM[6]. Larval enzyme solutions were freshly prepared before each test. Trypsin and α-chymotrypsin were commercial crystalline preparations obtained from Worthington Biochemical Corporation. C_1 was prepared from ether-extracted soybean flour (Lincoln var.) according to LIPKE *et al.*[1].

An attempt to fractionate C_1 (14.4% N) on a DEAE-cellulose column resulted

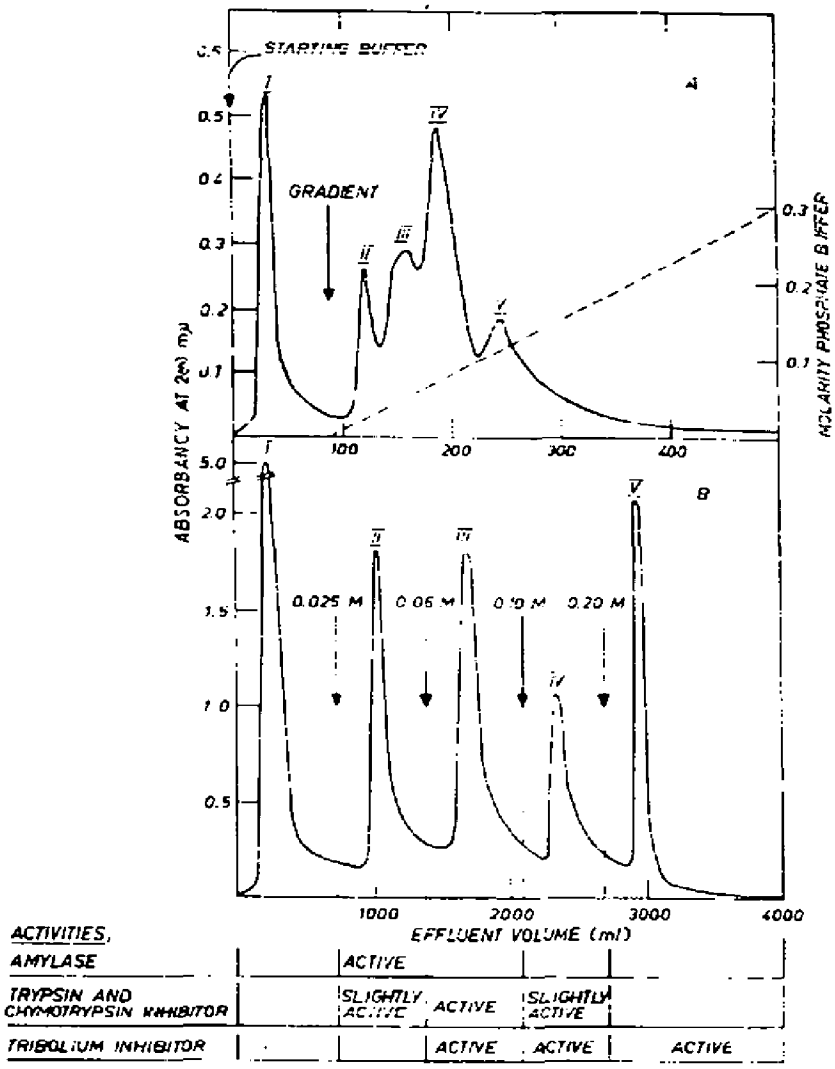

Fig. 1. Chromatographic pattern of C_1 on a calcium phosphate column. A, gradient elution with phosphate buffer (pH 6.8); B, stepwise elution with phosphate buffer (pH 6.8).

in a partial separation of the Tribolium inhibitor from the trypsin inhibitor, the former being still accompanied by some activity of the latter. Separation was not improved by changing the pH and the ionic strength of the eluting buffer. C_1 was then applied to a calcium phosphate column (hydroxylapatite), prepared according to TISELIUS *et al.*[7]. The column (1.7 × 6 cm) was charged with 60 mg of C_1 in 6 ml 1 mM phosphate buffer (pH 6.8). Gradient elution was performed with phosphate buffer (pH 6.8), 0.001 M → 0.3 M (mixing chamber volume 400 ml) at room temperature. Five distinct protein peaks were obtained (Fig. 1A), the Tribolium inhibitor being present in Fractions III, IV and V. Only Fraction V was free of trypsin and chymotrypsin inhibitor and of amylase activity. For preparative purposes gradient elution was replaced by elution with 4 stepwise increases of buffer concentra-

tion, *i.e.*: 0.001 M, 0.03 M, 0.06 M, 0.10 M, and 0.20 M phosphate buffer (pH 6.8). C_1 (2 g in 50 ml starting buffer) was applied to a 3.6 × 17.5 cm calcium phosphate column. The flow rate was adjusted to 100 ml/h and 8-ml fractions were collected. All operations were carried out at room temperature. The protein content of each tube was evaluated by measuring the ultraviolet absorption at 280 mμ and effluent fractions were examined for trypsin-, α-chymotrypsin- and Tribolium-inhibiting activities, as well as for amylase activity. The distribution of protein and of activities in the effluent fractions is shown in Fig. 1B. About 95% recovery of the protein was achieved.

Fig. 1B shows that the Tribolium inhibitor is present in Fractions III, IV, and V. However, only Fraction V is free of any amylase activity and of trypsin and chymotrypsin inhibitor. This Fraction V can completely inhibit the activity of *Tribolium castaneum* and *Tribolium confusum* larval protease, its specific activity being about

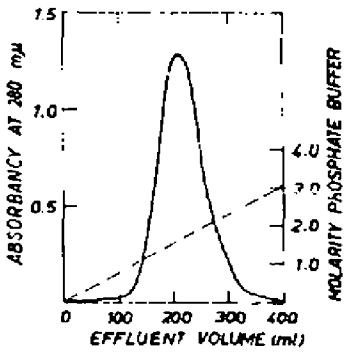

Fig. 2. Rechromatography of Fraction V. Linear gradient to 0.3 M phosphate buffer concentration is represented by broken line.

twice that of C_1. 5 μg of Fraction V per ml reaction mixture cause a decrease of 0.150 in absorbancy at 280 mμ, which corresponds to 50% inhibition of proteolysis under optimal experimental conditions. The same effect is achieved by 12 μg C_1/ml reaction mixture.

The chromatographic validity of Fraction V was established by its rechromatography, with gradient elution, on a calcium phosphate column (Fig. 2).

It may thus be hoped that this isolated proteineous fraction from soybeans, which inhibits Tribolium proteolytic enzymes specifically, will serve as a helpful tool in the study of insect proteolytic enzymes.

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Received August 2nd, 1962