INVESTIGATIONS ON FICIN

III. PURIFICATION OF FICIN BY GEL FILTRATION AND THE CHARACTERIZATION OF OTHER PROTEIN FRACTIONS OF FICUS LATEX

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After earlier success in the column chromatographic purification of ficin on CM cellulose and then DEAE cellulose[1], attention has now been turned to a technique permitting not only the purification of ficin, but also the characterization of other protein fractions present in Ficus latex, used as a proteolytic agent in certain countries[2]. The choice fell on gel filtration, an efficient technique for the separation of biologically active proteins[3].

EXPERIMENTAL AND RESULTS

Ten grams of a powder obtained in the crude form from the latex of *Ficus antihelmintica** were dissolved in 200 ml of water, 60 g of ammonium sulphate were added, and the solution was allowed to stand for 1 h at 5°. The resulting precipitate was separated off by centrifuging the solution at 10,000 r.p.m. for 10 min, washed three times with a 23% solution of ammonium sulphate, redissolved in 150 ml of water, and dialysed in a cold-room for 48 h with 10 l of distilled water. The resulting precipitate was removed by successive centrifuging, and, on being rendered lyophilic, the remaining clear solution gave about 5 g of pure sample.

50 mg of the sample were dissolved in 5 ml of a 0.005 *M* tris–phosphate buffer (pH 7.2, 0.9 % of NaCl), placed on a 90 × 3 cm column packed with Sephadex G-100, and eluted with the same buffer at an average flow rate of 30 ml/h. The eluent fractions were monitored spectrophotometrically for proteins, which showed an extinction maximum at 280 mμ (broken curve in Fig. 1A). A 400 μl portion of each fraction was then incubated at 37° for 1 h with 1 ml of a 1 % casein solution, the proteins were precipitated with 5 % trichloroacetic acid, centrifuged, and the supernatant liquid was used to determine the extinction at 280 mμ. The enzymatic activity of the various eluent fractions, found in this manner, is shown by the full curve in Fig. 1A.

Whereas the broken curve (protein concentration) exhibits five peaks (and an inflexion high up in the central peak), the fuli curve (enzymatic activity) stops after the large central peak coinciding with the third and highest maximum in the dashed curve, and, therefore, this is attributed to ficin.

Effect of temperature

To study the effect of temperature on the enzymatic activity of *Ficus* proteins,

* Purchased in Colombia by Prof. G. Navarro, whom we wish to thank.

a number of solutions were prepared, each from 50 mg of the lyophilized sample and 5 ml of 0.005 *M* tris–phosphate buffer (pH 7.2). The solutions were incubated at 37° for various periods, and then eluted down a 90 × 3 cm Sephadex G-100 column. After incubation for two or more hours, the enzymatic activity of the fraction corresponding to ficin is reduced to about 25 % of the initial activity (Fig. 1B).

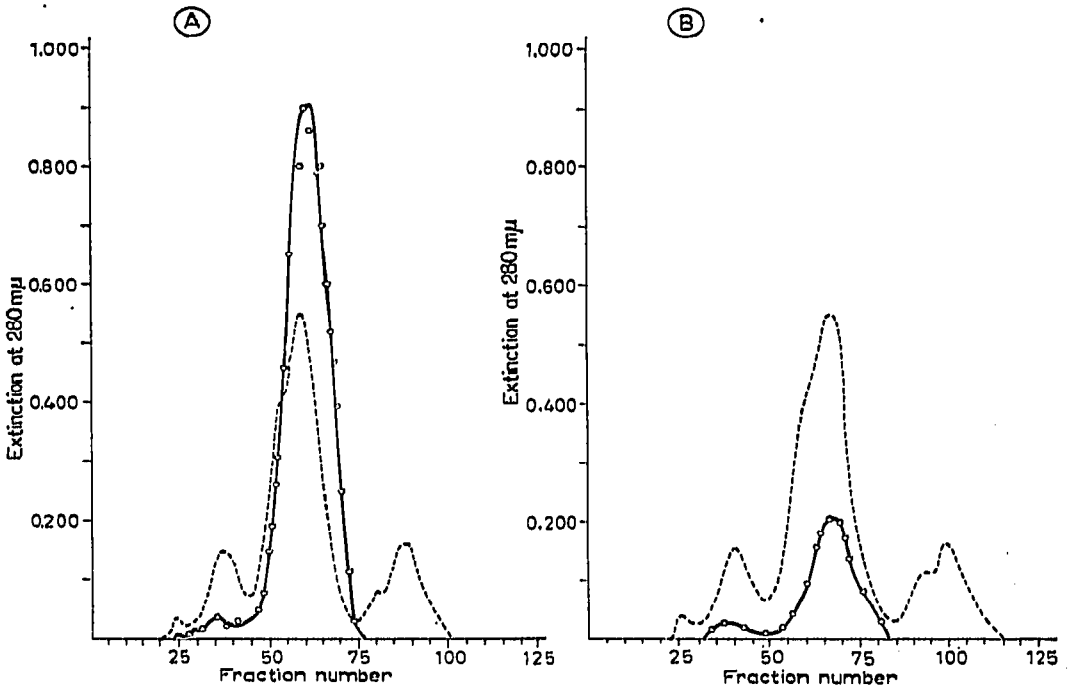

Fig. 1. (A) Extinction (at 280 mμ) of Ficus proteins separated on Sephadex G-100 (broken curve), and the corresponding enzymatic activity determined by Kunitz's casein method (full curve). (B) Enzymatic activity, after preincubation of the original protein mixture for 2 h at 37°. Extinction and enzymatic activity are indicated as in (A).

To find the cause of the inactivation of ficin, a 300 mg portion of the lyophilized mixture was first separated on Sephadex G-100, the resulting fractions were then individually incubated at 37° for various periods, and the enzymatic activity was finally determined at hourly intervals with the aid of 400 μl of casein solution. The results show that ficin almost entirely retains its activity even after 2 h at 37°. The inhibition observed in the previous experiment must therefore be due to a factor present outside the enzyme molecule, though still within the mixture.

Fig. 2 shows furthermore that incubation raises the enzymatic activity of the first two protein fractions F-1 and F-2. The question now is whether these contain ficin of other Ficus enzymes activated by incubation. To find out whether both F-1 and F-2 are complexes formed between a substrate and an enzyme that is activated by incubation and gives hydrolysates, 50 mg samples of F-1 and F-2 were dissolved in 5 ml of buffer and re-eluted through Sephadex G-100. A 400 μl portion of each eluent fraction was used to determine the enzymatic activity with casein, and the rest was kept at 37° for 5 h, treated with trichloroacetic acid, and centrifuged. The supernatant liquid containing the autolysates was used to determine the extinction at 280 mμ. The resulting curves (see Fig. 3) show the same variation in the enzymatic activity and in the autolysates. This indicates that each of the two protein fractions F-1 and F-2 is

composed of an enzyme and a substrate forming a higher-molecular-weight complex.

In order to confirm this result, 400 mg of F-1 and F-2 were fractionated on Sephadex G-100, the elution fractions were kept at 37° for 5 h, combined, dialysed, lyophilized, dissolved in tris–phosphate, and separated on Sephadex G-100. The protein determination was carried out by the Lowry-Folin method, the results being shown in Fig. 4. These peaks, the first two of which are very large, are probably

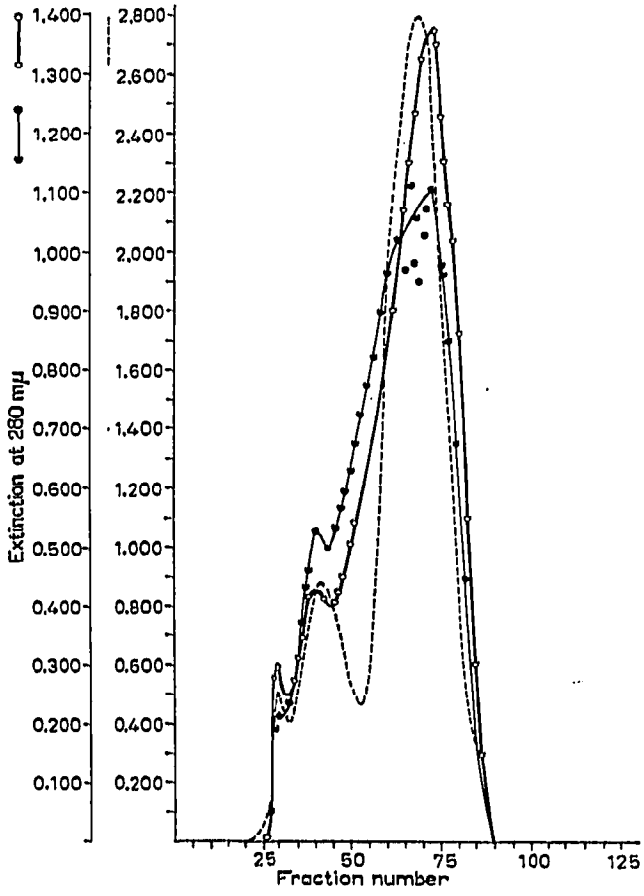

Fig. 2. Enzymatic activity without incubation of the fractions (o–o–o) and after incubation for 2 h at 37° (•–•–•). The protein concentration is shown by the broken curve.

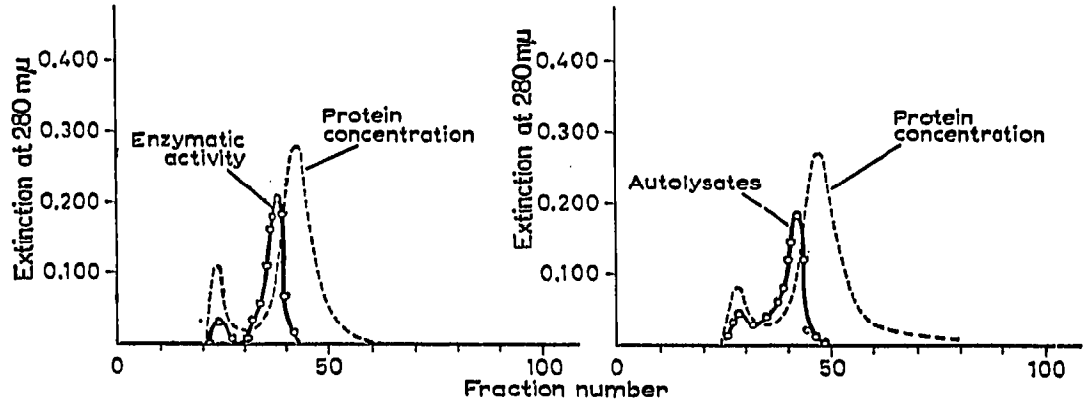

Fig 3. Correspondence between the enzymatic activity and the autolysates of the first two protein fractions shown in Fig. 1A.

ascribable to enzymatically active ficin and two inactive peptides. The protein fractions F-1 and F-2 are thus relatively stable, and cleavage leads to the same components as those found in nature. The curve for the enzymatic activity determined with casein and shown in Fig. 4 gives an even clearer representation of the process: a large activity maximum is found in the eluent volume corresponding to ficin, and since the enzyme is known to have been absent from the starting material used in this experiment, it must have been formed by the action of heat on the complex.

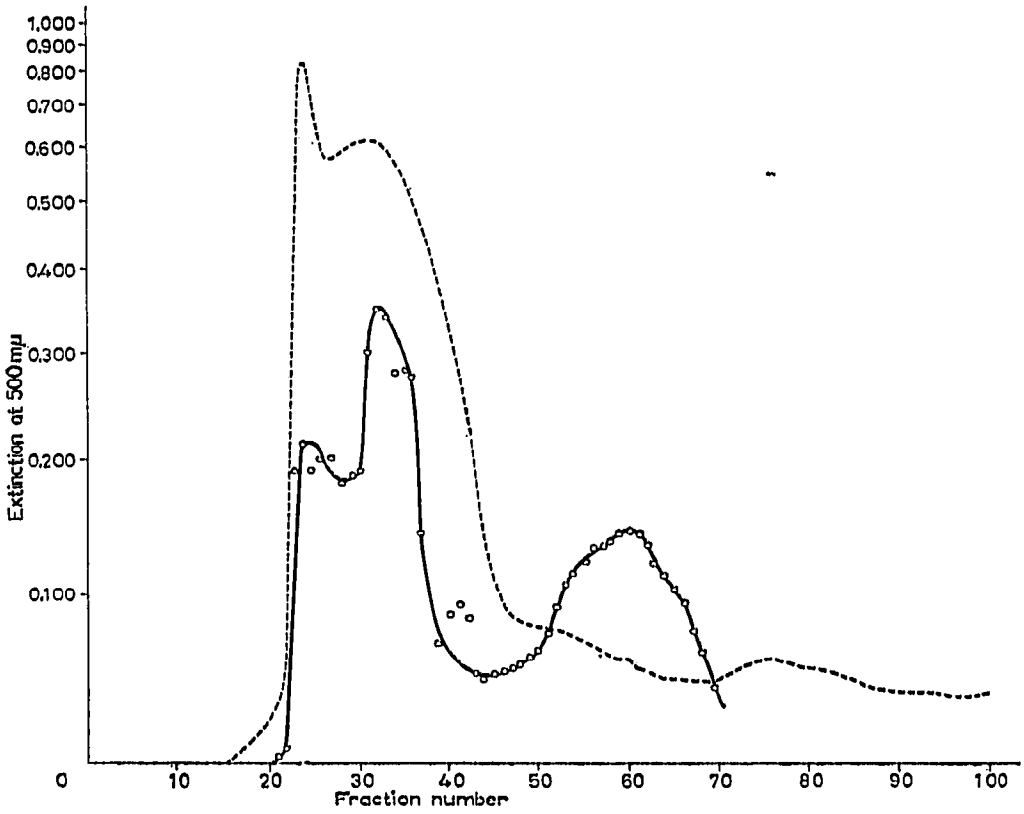

Fig. 4. Concentration of the two proteins F-1 and F-2 determined by the Lowry-Folin method (broken curve), and their enzymatic activity (full curve) determined with casein after autolysis. The thermal treatment gives rise to protein fragments, one of which coincides with the elution volume of enzymatically active ficin.

DISCUSSION

Sephadex G-100 is particularly useful for the separation of Ficus proteins extracted with ammonium sulphate, and represents considerable labour saving with respect to earlier techniques[1].

The presence of an inflexion in the ficin peak (Fig. 1) and the asymmetrical disposition of the enzymatic activity curve about the protein concentration curve suggest the existence of two enzyme forms—probably isoenzymes—differing in activity and also somewhat in molecular weight.

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SUMMARY

Purified ficin obtained by repeated precipitation with ammonium sulphate was passed through a column packed with Sephadex G-100.

Ficin and other protein fractions were obtained. The first two fractions, by heat effect (37°), give rise to proteolytic active fraction.

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