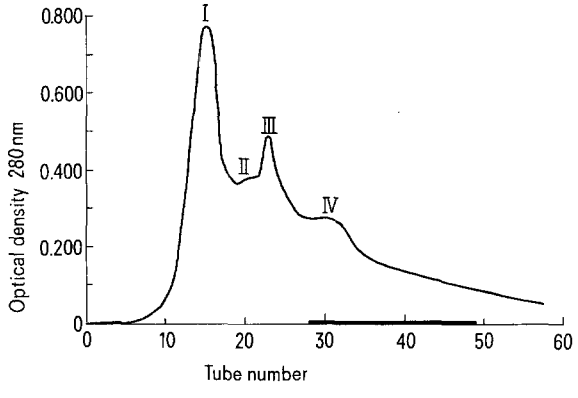
Inhibitory Peptides from Concanavalin A

SUMNER and HOWELL[1] in their classical paper on the hemagglutinin from the jack bean, concanavalin A (Con A), observed that it was rather resistant toward tryptic digestion, an observation later confirmed by AGRAWAL and GOLDSTEIN[2], who found that pronase, chymotrypsin, pepsin and papain destroyed the agglutinating activity of Con A more readily than trypsin.

The active tetrameric Con A molecule has 4 binding sites that combine with receptors on the susceptible cell surface[3]. In the present paper we propose to obtain by controlled proteolysis a partially degraded Con A molecule with one intact binding site. This peptide molecule should not possess hemagglutinating activity, but being able to combine with a receptor it should cause inhibition of the normal agglutination by Con A.

Material and methods. Con A was prepared from extracts of ground jack beans by the method of OLSON and LIENER[4]. After preliminary experiments on the yield of the inhibitory fraction by proteolytic degradation of Con A with several commercial proteases, the following method was chosen: to 30 mg Con A in 5 ml 0.066 *M* phosphate buffer, pH 7.5, 500 µg trypsin (bovine pancreas, 1 × crystallized, Sigma Chem. Comp., St. Louis, Mo.) in 0.1 ml 10^{-3} *M* HCl were added. Digestion time was 24 h at 25 °C, after which the digest was passed through a Sephadex G-10 column (0.8 × 50 cm) using bidistilled water as eluant. The effluent was collected in 1 ml fractions and optical density measured at 280 and 220 nm. Hemagglutinating activity was determined on pronase-treated rabbit erythrocytes using a microtitration kit (Cooke Engineering Comp. Alexandria, Va.). 25 µl of the peptide fractions (40–80 µg) to be tested were put into each well of the microtiter plate, 25 µl of Con A (5 µg) were added to the first well and diluted in geometrical sequence in each of the following wells. Finally, 25 µl of a 4% pronase-treated rabbit erythrocyte solution were added and hemagglutination observed after 30 min at 25 °C.

In some experiments, instead of native, denatured Con A was used for the obtention of the inhibitory peptides. The following procedure of denaturation was used: 30 mg Con A dissolved in 2.5 ml 0.2 *M* carbonate buffer, pH 10.2, were kept at 50 °C for 10 min, cooled immediately to 25 °C, neutralized with 1 *M* HCl and brought to 5 ml final volume with 2.3 ml 0.066 *M* phosphate buffer, pH 7.5. This solution was devoid of hemagglutinating activity.



Chromatography of Con A-trypsin-digestion products on a Sephadex G-10 column. The fractions marked by the bar were pooled and freeze-dried. The pool inhibited hemagglutination of rabbit erythrocytes by Con A.

The action of the peptide fraction on the mitogenic activity of Con A was measured by the incorporation of ^{3}H-thymidine into cultured human lymphocytes[5].

Results. The results of the Sephadex G-10 chromatography of the trypsin digestion products are depicted in the Figure. Peak I contained active Con A, when the native protein was digested at 25 °C for 18 h. When digestion was performed at 37 °C or when denatured protein was used, this eluted material was devoid of hemagglutinating activity.

Peak IV contained the material capable of inhibiting the hemagglutinating and mitogenic activities of native Con A. Inhibition by peak IV was observed when either native or denatured Con A had been used for digestion (Table). The yield of peak IV was about 14% and was the same in both cases. When tryptic digestion was extended to 48 h, the inhibitory activity was lost. Also, digestion of peak IV with pronase for 18 h at pH 7.5 completely abolished its inhibitory activity on Con A. Included in the Table are the results of an experiment showing that the hemagglutinating and mitogenic activities of the kidney bean hemagglutinin are not inhibited by fraction IV.

Discussion. The results show that by limited proteolytic degradation of Con A, native or denatured, products are obtained which are capable of inhibiting hemagglutination and mitosis induced by native Con A.

These products are of peptide nature and consist, probably, of 'monovalent' molecules, in which one active site of Con A has an unchanged configuration and therefore blocks a cell receptor. Chromatography of the inhibitory fraction on a Sephadex G-75 column yields several active peptides eluting between the 10 and 20,000 molecular weight markers, two of which are present in relatively large amounts. The retention of these rather large peptides on Sephadex G-10 must be due to their hydrophobic interaction. High voltage paper electrophoresis of the digest showed 6 rather hydrophobic peptides, which supports the previous hypothesis. These inhibitory peptides can be obtained from native or alkali-treated inactive Con A. It seems, therefore, that alkali denaturation did not affect the configuration of the zone responsible for the interaction with cell receptors. The fact that these peptides inhibit both hemagglutinating and mitogenic activities suggests that in the intact Con A molecule more than one active site is required, not only for hemagglutination[3] but also for mitogenic action. From the lack of inhibitory activity toward bean hemagglutinin, it can be concluded that the cell receptors for the latter are different form the ones binding Con A. Digestion products from Con A possessing inhibitory activity have been described before and their action on cultured cells studied[6,7], but no further characterization has been reported.

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Comparison of the inhibitory action of fraction IV on hemagglutination and mitosis produced by Con A.

Agglutinin	Peptide fraction	Agglutination[a] titer	Cpm[b]	Incorporation (%)[c]
Con A	None	4	9.981	100
None	I (from native Con A)	4	7.993	80
None	I (from denatured Con A)	0	180	2
None	II (from native Con A)	0	220	2
None	III (from native Con A)	0	230	2
None	IV (from native Con A)	0	243	2
Con A	I (II or III)	4	9.700	97
Con A	IV (from native Con A)	0	2.240	22
Con A	IV (from denatured Con A)	0	3.230	32
Con A	IV Pronase-treated	4	9.043	91
Con A	IV Trypsin-treated 48 h	4	—[d]	—
Bean PHA	IV (from native Con A)	4	16.850	91
Bean PHA	None	4	18.500	100

[a] Measured as described in text. [b] Counts per min incorporated ^{3}H-thymidine in cultured human lymphocytes. [c] Percent incorporation of ^{3}H-thymidine as compared to the one produced by Con A. [d] Not measured.

The inhibitory fragments from Con A are promizing tools for the further investigation of the role played by Con A receptor sites of normal and transformed cells. Probably, similar inhibitory peptides can be obtained from other phytohemagglutinins and can be used to study the different aspects of their biological activities[8].

Zusammenfassung. Durch Trypsinverdauung von nativem und denaturiertem Concanavalin A wurde eine Peptidfraktion erhalten, die sowohl die hämagglutinierende als auch die mitogene Wirkung von Concanavalin A hemmt. Die Fraktion zeigte jedoch keine Wirkung auf die Aktivität von Bohnenagglutinin.

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Electrophoretic Study of Carboxylesterases During the Ontogenesis of *Medicago scutellata*

During differentiation, it is known for a number of enzymes that ontogenesis is associated with alteration in the isoenzyme content of the organisms. The differential synthesis of isoenzymes in cells may be considered as an elementary process in cell differentiation[1]. Support for the concept of differential gene activation during cellular differentiation has been derived from studies of the ontogenesis of isoenzymes. Such studies in the case of carboxylesterases have been carried out mainly in animals[2–9], while no complete study has yet been made on the ontogenesis of carboxylesterases in plants[10–13]. The purpose of this study was to investigate whether multiple molecular forms of carboxylesterases occured in a wild species of Alphalpha (*Medicago scutellata*), and whether these forms were changed during the development of the plant.

Material and methods. Seeds of *Medicago scutellata* (Miller) were collected from Patras fields, disinfected with a 25% solution of commercial liquid bleach ('Klinex') and part of the seed-coat was removed in order to facilitate germination. The seeds were allowed to germinate at 25 °C in petri dishes lined with filter paper wetted with water. After the germination, the seedlings were grown in pots. Samples from different organs were taken at various developmental stages, homogenized with distilled water (1:5 W/V), filtered through cheese-cloth, centrifuged for 30 min at 30,000 *g* at 4 °C and the supernatant was collected. Horizontal starch gel electrophoresis was carried out using the discontinuous buffer of ASHTON and BRADEN[14] with some modifications (the gel buffer was diluted to $^1/_3$ with distilled water), and a voltage gradient of 20 V/cm for $2^1/_2$–3 h. After electrophoresis, the gels were sliced horizontally and the carboxylesterase

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