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Phytochemistry, 1978, Vol 17, pp 546–548. Pergamon Press. Printed in England

AMINOPEPTIDASE ACTIVITY ASSOCIATED WITH ALPHA$_1$-CONARACHIN (PEANUT PROTEIN)

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(*Revised received* 19 *August* 1977)

Key Word Index—*Arachis hypogaea*, Leguminosae; peanut, aminopeptidase; enzyme adsorption, immunochemistry; germination.

Abstract—Aminopeptidases were investigated in protein extracts of dormant and germinated peanut cotyledons by electrophoretic immunochemical techniques. Considerable activity was observed in protein zones that migrated toward the anode after simple electrophoresis. Of the immunogenic proteins in dormant seed, aminopeptidase activity was associated only with the immunocomplex of α_1-conarachin, a globular protein in peanuts. The specific aminopeptidase activity of total extracts was marginally higher than that of purified α-conarachin. Specific isoenzyme(s) adsorption on the antigen–antibody complex of α_1-conarachin might be attributed in part, to these phenomena. Reactions of protein extracts from germinated cotyledons with immune sera made against protein in germinated tissues—roots and cotyledons—showed the identical associative interaction. Some of the determinant groups on α_1-conarachin in the germ and cotyledon were apparently maintained during early phases of germination.

INTRODUCTION

Aminopeptides in dormant seeds of peanuts have been investigated in our laboratory during the last few years [1, 2]. With starch gel electrophoresis, 4 isoenzymes that hydrolyzed L-leucyl-beta-naphthylamide-HCl were detected at the anode [2]. With polyacrylamide disc electrophoresis, 5 similar enzyme bands that hydrolyzed the same substrate were observed [1]. In these studies, simple electrophoresis was employed on whole extracts that contained mixtures of albumins and globulins of the peanut seed. The objective of the present study was to further investigate this enzyme system in both dormant seed and in tissues of the growing plant by the more specific immunochemical methods.

RESULTS AND DISCUSSION

The immunoelectrophoretic analysis (IEA) of peanut proteins soluble in buffered aqueous media, reported by Daussant *et al.* [3], showed that at least 14 precipitin arcs were detected with immune serum made from a total cotyledonary extract. Alpha-conarachin, the major component in the classic conarachin fraction isolated by ion-exchange chromatography, showed 2 serologically distinct proteins that were named α_1- and α_2- conarachin. Alpha$_1$-Conarachin migrated toward the anode and α_2-conarachin moved toward the cathode. In part, the present data are based on these results.

Preliminary analyses by simple electrophoresis in agar showed considerable aminopeptidase staining on poorly separated protein zones (not shown). Further analyses by IEA, however, showed activity only on one precipitin line that was located in the region where α_1-conarachin migrated. Therefore, simultaneous analyses using anti-α-conarachin and anti-cotyledonary proteins were conducted with purified α-conarachin and with total cotyledonary extracts (Fig. 1). These results showed enzyme activity associated only with the immunocomplex of α_1-conarachin (a), as evidenced by the coalescence of the 2 arcs. With purified α-conarachin (Con) as antigen, identical results were noted (lower Fig. 1a).

To further investigate this system, sera made against protein from germinated tissues—cotyledons, roots and leaves—were employed for qualitative and semi-quantitative analyses. In Fig. 1b, (double diffusion) immune sera from 5-day germinated cotyledons and roots, mature leaves, and α-conarachin were reacted with proteins of 7-day germinated cotyledons in series. The characteristic aminopeptidase reaction of identity was observed in all tissues except for the leaves. This was expected for leaves, since α_1-conarachin is not present in that tissue.

Enzyme staining on the immunochemical complexes of α_1-conarachin after progressive germination showed about the same intensity (Fig. 1c), however, some

* One of the facilities of the Southern Region, Agricultural Research Service, U.S Department of Agriculture.

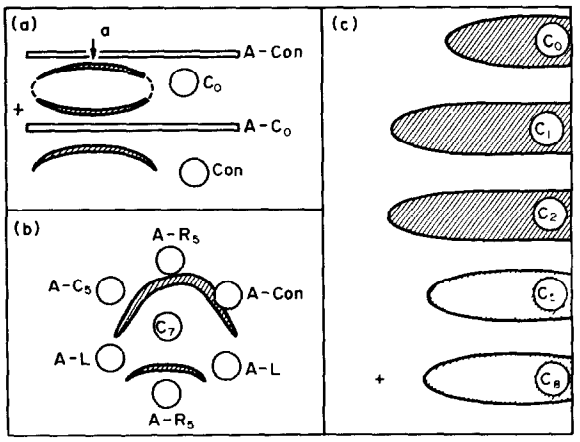

Fig. 1. Detection of aminopeptidase activities in immunocomplexes of peanut proteins. In (a) 0.5 mg of purified α-conarachin (Con) and of total cotyledonary proteins, (C_0), was applied in the indicated wells for immunoelectrophoresis. After electrophoresis, immune sera made against α-conarachin ((a) con) and against a total cotyledonary extract ((a) C_0) were placed in the troughs. Identification of α_1-conarachin (a) is indicated. In (b) the center well contained 0.5 mg protein from a 7-day germinated cotyledonary extract (C_7); the surrounding wells contained immune sera anti-5-day cotyledons ((a) C_5), anti-5-day root ((a) R_5), anti-α-conarachin ((a) con), and anti-mature leaf ((a) L). In (c) 80 μg of protein from extracts of cotyledons following germination up to 8 days (C_0 to C_8) was applied in each well prior to electrophoresis; 5% immune serum, anti-5-day cotyledons, was dispersed in the agar. All agar plates were washed with saline solutions to remove unreacted serum proteins and excess peanut proteins, and dried before development of aminopeptidase reactions.

differences with respect to electrophoretic mobility were observed. The length of the conical peaks formed is directly proportional to concentration of antigen, provided the number of active determinant groups and net charges on the antigens under investigation are identical. Therefore, a high peak may be due to either a high concentration of antigen, to low antigenicity, or to increased negative charge of a specific protein [4]. The number and types of determinant groups on α_1-conarachin are not known. Hence, based on equal quantities of protein in each well, only semi-quantitative interpretation is feasible. The slight anodic shift in migration that reached a maximum on the 2nd day of germination and which was reduced slightly thereafter could be attributed to structural alterations that reflect changes in net charge and/or active determinant groups on α_1-conarachin. One major point here is that the apparent maintenance of native determinant groups on α_1-conarachin indicated slow hydrolysis during germination, due to peptidase activity which remained associated with it.

The specific aminopeptidase activity of a total cotyledonary protein extract from dormant seed was 1.1 mkat/mg protein. For purified α-conarachin, the specific activity was 0.9 mkat/mg protein. Alpha$_1$-conarachin represents less than 20% of the total proteins in peanuts [5] and yet the activity is marginally higher in the toal extract. Undoubtedly, not all of the aminopeptidase activity can be attributed to α_1-conarachin, considering the number of isoenzymes previously shown to exist in total cotyledonary extracts [1, 2] and the intense staining observed in the preliminary experiments.

As mentioned earlier, the existence of multiple forms of aminopeptidases in peanuts has been established [1, 2]. From the present data, part of that activity was associated with one of the previously characterized globulins, α_1-conarachin. Judging from the marginal difference in specific aminopeptidase activity of this isolated protein and that of a total cotyledonary extract, however, these data suggest that one or more isoenzymes of aminopeptidase could be adsorbed specifically to α_1-conarachin without inhibiting immunoprecipitation. Whatever the mechanism of adsorption, it was obviously maintained after purification of α_1-conarachin and persisted in cotyledons during early phases of germination. The fact that α_1-conarachin in cotyledons retained some active antigenic determinant groups during growth and apparently remained intact in young roots suggested that it is not a major source of nitrogen for protein synthesis during early phases of growth. An earlier study by Daussant *et al.* [6] showed that α-arachin, the major peanut globulin, was partially hydrolyzed after the first day of germination.

Obviously, the data presented here are not adequate to explain the adsorption phenomenon and to ascertain to what extent α_1-conarachin accounts for the observed aminopeptidase activity. This information, however could be useful in correlative studies of aminopeptidases in different varieties of peanuts in the dormant state and during germination.

EXPERIMENTAL

Extraction and purification of protein. Proteins in tissues of dormant and germinated cotyledons, roots, and leaves were extracted at 5° in NaPi buffer (8 mM NaH_2PO_4, 64 mM Na_2HPO_4), pH 7–8, ionic strength 0.2 (1 g tissue per 2 ml of buffer). The homogenates were warmed to 25°, filtered through several layers of cheesecloth and the filtrate centrifuged at 38000 *g* for 20 min at 20°. The lipid layers and pellets were discarded and the remaining liquid fractions were recentrifuged under the same conditions to remove most of the lipids. The extracts were frozen for storage. Alpha$_1$-conarachin was purified by linear gradient elution chromatography on DEAE-cellulose as reported in ref. [3]. Protein in each sample was determined by the method of ref. [7], or by the Kjeldahl method.

Germination of seeds. One-year-old select peanut seed (variety 56R) were obtained from the Agricultural Research Service, Crops Research Division, Beltsville, Maryland. The seeds were surface-sterilized for 15 min in 10% Clorox, rinsed, soaked 2 hr in H_2O and germinated in moist vermiculite. The plants were grown under glass at 25° in diffuse sunlight and natural photoperiod. At selected stages, the cotyledons or roots were excised, washed, blotted dry and extracted as described in the first section. Leaf protein was obtained from mature plants that were grown in the field.

Immunochemistry and enzyme assays. Immunoelectrophoresis and simple electrophoresis were carried out according to ref. [8] in 1.5% Ionagar gel (Oxoid, Ltd., London; electrophoresis was carried out in 25 mM veronal buffer, pH 8.2 with a voltage gradient of 4 V/cm for 2 hr at room temp. Electroimmunodiffusion was conducted in the above veronal buffer according to ref. [9] in 1.5% Ionager, employing 300 V and 30 mA for 3 hr at room temp. The principle involves the electrophoretic migration of an antigen that forms a solid

immunochemical complex with antibody embedded in agar gel. Double diffusion of antigen/antibody solns was performed according to ref. [10] in 1.5% Ionagar. Qualitative aminopeptidase activities on the immunoprecipitates were detected by incubating the dried plates for 2 hr in 100 ml of 0.2 M Pi buffer, pH 4.4, containing 20 mg of L-leucyl-beta-naphthylamide-HCl as substrate, and 25 mg of Black Salt K. This technique, described in ref. [11], is particularly useful in identification procedures within a mixture of enzymes or other proteins. The immune sera were prepared by injections in 3 rabbits of proteins in appropriate fractions, according to the standard procedure of Antibodies Incorporated, Davis, California. Specific aminopeptidase activity was determined by the method of ref. [12]. Activities are expressed as katals per mg protein; a unit is defined as one mol of substrate hydrolyzed per sec at 30°.

Acknowledgements—The determinations of sp. act. of aminopeptidases and discussions of the subject matter with Dr Juhani Mikola, Department of Biology, University of Jyväskylä, Jyväskylä 10, Finland, and the line drawing of Nancy Meadow, are gratefully acknowledged.

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Phytochemistry, 1978, Vol 17, pp 548–549 Pergamon Press Printed in England

SPECIFICITY OF ENZYME SYSTEM PRODUCING C_6-ALDEHYDE IN *THEA* AND *FARFUGIUM* CHLOROPLASTS

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(*Received* 31 *May* 1977)

Key Word Index—*Thea sinensis*, Theaceae; *Farfugium japonicum*, Compositae; isolated chloroplasts; lipoxygenase; *cis*-3-hexenal; *n*-hexanal; *cis*-3, *cis*-6-dienoic acids (C_8–C_{12}); substrate specificity

Recently it was confirmed that linolenic acid is split into C_6-aldehyde, *cis*-3-hexenal, and C_{12}-oxo-acid, 12-oxo-*cis*-9-dodecenoic acid, by an enzyme system in chloroplasts of tea leaves, using linolenic acid-[^{14}C] [1, 2]. The enzyme system in tea chloroplasts catalyzes the oxidative splitting of C_{18}-unsaturated fatty acids through addition of oxygen to the double bond at C-12 and produces C_6-aldehydes. The structural requirement for substrates is the presence of a free carboxyl group and a *cis*-1, *cis*-4-pentadiene system including the double bond at C-12 [3]. *Farfugium japonicum* chloroplasts have also given a similar result to that of tea chloroplasts [4].

This report describes the substrate specificity of an enzyme system producing C_6-aldehydes for a series of synthesized *cis*-3, *cis*-6-dienoic acids in which the chain length varies from C_8 to C_{12} [5].

As shown in Table 1, when *cis*-3, *cis*-6-nonadienoic acid was used as a substrate, 40 and 67% of C_6-aldehyde (*cis*-3-hexenal) were produced by tea and *F. japonicum* chloroplasts, respectively, compared to *n*-hexanal formation from linoleic acid. *cis*-3, *cis*-6-Dodecadienoic acid gave 21 and 11% of C_6-aldehyde (*n*-hexanal). Neither acid (C_9 and C_{12}) gave any other volatile aldehydes (e.g. C_9-aldehydes). The other acids (C_8, C_{10} and C_{11}) were poor substrates or gave no expected aldehydes. On the other hand, these *cis*-3, *cis*-6-dienoic acids (C_8–C_{12}) did not act as substrates for soybean lipoxygenase.

Table 1. Substrate specificity of the enzyme system producing C_6-aldehydes

Substrate	Relative activity (%)		Product
	Tea	*F. japonicum*	
cis-3, *cis*-6-dodecadienoic acid	21	11	*n*-hexanal
cis-3, *cis*-6-undecadienoic acid	0	0	
cis-3, *cis*-6-decadienoic acid	0	0	
cis-3, *cis*-6-nonadienoic acid	40	67	*cis*-hexenal
cis-3, *cis*-6-octadienoic acid	0	<3	

Activities are expressed relative in mole number to *n*-hexanal obtained with linoleic acid.

It is concluded that *cis*-3, *cis*-6-nonadienoic acid and *cis*-3, *cis*-6-dodecadienoic acid, which have a double bond of *cis*-1, *cis*-4-pentadiene system at the 6th carbon atom counting from the terminal methyl group, act as a substrate for the enzyme system producing C_6-aldehydes in chloroplasts, though they have a smaller number of carbon atoms than linolenic and linoleic acids.