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DE NOVO SYNTHESIS AND HORMONAL REGULATION OF A DIPEPTIDASE IN *CUCURBITA MAXIMA*

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Key Word Index—*Cucurbita maxima*; Cucurbitaceae; squash; dipeptidase; hormonol regulation; gibberellic acid; benzyladenine; indol-3-acetic acid; abscisic acid.

Abstract—The following evidence was obtained for the *de novo* synthesis of dipeptidase in squash (*Cucurbita maxima* Duch. var. Hubbard) cotyledons during germination: (i) the amount of [^{14}C]leucine incorporated into the dipeptidase was greater than that found in other proteins; (ii) the enzyme coincided with a peak of radioactivity in DEAE column chromatography; and (iii) the specific radioactivity of the enzyme increased with purification. There was also a positive correlation between the rate of [^{14}C]leucine incorporation into dipeptidase and the rate of dipeptidase development. Four plant growth regulators, gibberellic acid (GA), benzyladenine (BA), indol-3-acetic acid (IAA), and abscisic acid (ABA) were examined for their effect on the development of dipeptidase activity at 5×10^{-6} and 5×10^{-5} M. None of these regulators affected the activity of the isolated dipeptidase *per se*. In intact seeds, BA and IAA inhibited the development of dipeptidase activity at the higher concentration, ABA reduced the activity at both concentrations; however, GA enhanced its development at the higher concentration. In distal-half cotyledons, BA and GA stimulated enzyme development but they showed no synergistic effect. IAA suppressed the development of enzyme activity at the higher concentration and ABA inhibited development at both levels.

INTRODUCTION

THE INCREASE in dipeptidase activity of squash cotyledons was strongly inhibited by cycloheximide, and *de novo* synthesis was suggested.[1] However, the inhibition of an increase in the enzyme activity by a protein synthesis inhibitor may be the result of an inhibition of the synthesis protein that is required for the activation of an enzyme, rather than the inhibition of the *de novo* synthesis of the enzyme.[2-4] Shain and Mayer have shown that protein synthesis inhibitors partially inhibit the activation of a trypsin-like enzyme in lettuce seeds[3] and amylopectin-1,6-glucosidase in pears.[4]

Squash seeds required the presence of the embryo axis for maximum development of dipeptidase activity[1] and benzyladenine reproduces the effect of the embryo axis. A proteinase, isolated from the same tissue, was also controlled by cytokinins but not by GA or IAA.[5] The dipeptidase under study has been purified and characterized from squash cotyledons,[6] and the purpose of this investigation was to determine whether the dipeptidase is synthesized *de novo* and whether it is subject to hormonal control.

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TABLE 1. PURIFICATION OF DIPEPTIDASE FOLLOWING [^{14}C]LEUCINE FEEDING FOR 24 hr AFTER A 4-DAY GERMINATION PERIOD

Fraction	Volume (ml)	Protein (mg/ml)	Enzyme activity (units/ml*)	Specific enzyme activity (units/mg protein)	Radioactivity (cpm/ml)	Specific radioactivity (cpm/mg protein)	Purification (fold)	Recovery (%)
Crude (I)	100·0	48·50	3·81	0·079	3850	79·4	1·00† 1·00‡	100·0§ 100·0‖
Acetone (II)	13·2	17·80	11·04	0·620	4030	226	7·85† 2·85‡	38·2§ 13·8‖
Sephadex (III)	61·6	1·83	3·12	1·705	666	364	21·6 † 4·6 ‡	50·4§ 10·7‖
DEAE (IV)	114·0	0·075	3·76	50·1	45	600	635 † 7·56‡	113 § 1·3‖

* One unit = 1 ml of 0·1 M KOH/60 min.
† Enzyme purification: specific enzyme activity ÷ by specific enzyme activity of crude fraction.
‡ Radioactive purification: specific radioactivity ÷ by specific radioactivity of crude fraction.
§ Enzyme recovery: vol. × enzyme activity ÷ by volume × enzyme activity of crude fraction × 100.
‖ Radioactive recovery: volume × radioactivity ÷ by volume × radioactivity of crude fraction × 100.

RESULTS AND DISCUSSION

De novo *Synthesis*

The results of this study support the previous suggestion[1] that the dipeptidase is synthesized *de novo*. This is based on the fact that [^{14}C]leucine is incorporated into the dipeptidase to a greater degree than into other proteins, as shown by the pattern of ^{14}C in the DEAE fraction. Moreover, the specific radioactivity of the protein increased as the purity of the enzyme increased (Table 1). It has been previously shown by electrophoresis and ultracentrifugation that fraction IV contains only one protein, namely the dipeptidase.[6] The dipeptidase was purified 635-fold and the specific radioactivity, cpm/mg protein, increased 7·6-fold (Table 1).

There was a marked increase in the per cent recovery of dipeptidase activity between fraction II and fraction IV during purification. This indicates that an inhibitor was removed during purification and confirms the previous reports[1,6] concerning dipeptidase inhibitors in squash cotyledons.

Hormonal control, GA and BA

The development of dipeptidase activity in cotyledons of intact seeds or distal-half cotyledons was determined 4 days after germination (Table 2). It has been reported that the

TABLE 2. EFFECT OF DIFFERENT HORMONES ON THE DEVELOPMENT OF DIPEPTIDASE ACTIVITY DURING 4 DAYS OF GERMINATION. Values are expressed as enzyme units* per distal-half cotyledon

Amount added	Dipeptidase activity BA	GA	IAA	ABA
Intact seeds				
Control	0·393 *bc*†	0·456 *d*	0·348 *a*	0·468 *a*
5×10^{-6} M	0·349 *cd*	0·426 *d*	0·331 *ab*	0·354 *b*
5×10^{-5} M	0·262 *d*	0·529 *c*	0·253 *bc*	0·197 *c*
Distal halves				
Control	0·276 *d*	0·348 *c*	0·260 *bc*	0·336 *d*
5×10^{-6} M	0·461 *b*	0·643 *b*	0·176 *c*	0·339 *b*
5×10^{-5} M	0·621 *a*	0·795 *a*	0·182 *c*	0·231 *c*

* One unit = 1 ml of 0·1 M KOH/60 min.
† Means followed by the same letter are not significantly different at the 5% level as determined by Duncan's Multiple Range Test. All comparisons are made within the same column.

axial tissue is necessary for the development of maximal dipeptidase activity and the axis may supply a cytokinin for this response.[1] The data in Table 2 show that both BA and GA stimulate dipeptidase development in distal-half cotyledons at 5×10^{-6} M and at 5×10^{-5} M with greater increases at the higher concentration. Therefore, GA, as well as BA, produce the effect of embryo axis. The action of BA and GA on the dipeptidase activity is not known; however, both hormones have been reported to enhance nucleic acid synthesis.[7]

With intact seeds, the two hormones showed different effects on dipeptidase activity. At 5×10^{-6} M neither hormone significantly altered the dipeptidase activity (Table 2). However, at 5×10^{-5} M, GA promoted the enzyme activity slightly, whereas BA inhibited it. BA inhibition of dipeptidase activity in intact seeds has been previously observed by Sze and Ashton.[1] They suggested that it was the result of optimal amounts of endogenous BA being present in the intact seeds and additional BA was therefore inhibitory.

TABLE 3. EFFECT OF COMBINATION OF BA AND GA ON THE DEVELOPMENT OF DIPEPTIDASE ACTIVITY DURING 4 DAYS OF GERMINATION. Distal-half cotyledons were used in the experiment. Values are expressed enzyme units* per distal-half cotyledons

Treatment	Dipeptidase activity
Control	0·230 *e*†
GA 5×10^{-6} M	0·514 *d*
BA 5×10^{-6} M	0·531 *cd*
GA 5×10^{-5} M	0·607 *bcd*
BA 5×10^{-5} M	0·709 *ab*
GA 5×10^{-6} M + BA 5×10^{-6} M	0·570 *bcd*
GA 5×10^{-6} M + BA 5×10^{-5} M	0·682 *abc*
GA 5×10^{-5} M + BA 5×10^{-6} M	0·545 *bcd*
GA 5×10^{-5} M + BA 5×10^{-5} M	0·783 *a*

One unit = 1 ml of 0·1 M KOH/60 min.

† Means followed by the same letter are not significantly different at 5% level as determined by Duncan's Multiple Range Test.

Interaction of GA and BA

This experiment was designed to determine the interaction of GA and BA on the development of dipeptidase. Two concentrations of BA were combined with two concentrations of GA. Table 3 shows that there was no significant additive or synergistic responses to the combinations. This suggests that both hormones are influencing the same type of response on the development of dipeptidase activity, perhaps through nucleic acid metabolism. GA has been reported to induce the synthesis of α-amylase, protease, and ribonuclease.[7–10] This is considered to be the result of GA-induced RNA synthesis.[11,12] It has been suggested that BA induces the synthesis of dipeptidase, proteinase, and isocitrate lyase.[1,5,13,14] BA also increased the ^{32}P-labeling of all nucleic acid species.[12] Kinetin has

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been shown to increase the label of nuclear RNA and to increase the [^{14}C]adenine labeling of RNA.[15,16] Kinetin also increased the RNA content of tissues.[17] Therefore, GA and cytokinins appear to promote the synthesis of dipeptidase through increased nucleic acid synthesis.

Hormonal control, IAA and ABA

IAA has been reported to be incapable of causing an increase in proteinase activity in squash cotyledons.[5] In our study, IAA at 5×10^{-6} M did not affect dipeptidase activity but at 5×10^{-5} M it inhibited the activity in intact seeds (Table 2). In distal-half cotyledons, IAA appears to inhibit dipeptidase activity at both concentrations. In another study, 2,4-D, a synthetic hormone which is similar to IAA in action, at 10^{-5} M decreased the dipeptidase activity in the same plant material.[18] The effect of IAA and other auxins on nucleic acid metabolism has been investigated extensively.[19,20] In general, concentrations of auxins that inhibit growth also inhibit nucleic acid and protein synthesis while concentrations that stimulate growth promote the synthesis of nucleic acid and proteins.

In intact seeds, ABA at 5×10^{-6} M, reduced the dipeptidase activity about 25% and at 5×10^{-5} M it was decreased about 60% (Table 2). In distal-half cotyledons, only the higher concentration of ABA inhibited the enzyme development. These results indicate that in the intact seed system, the lower concentration of ABA prevented the influence of embryo axis and at the high concentration inhibitions greater than the embryo axis influence occurred. The action of ABA has been recently reviewed.[21] The inhibitory effects of ABA on germination and growth are correlated with inhibitory effects in enzymes, i.e. α-amylase, protease and ribonuclease.[7,22,23] Nucleic acid synthesis is also inhibited by ABA.[21] The kinetics of the interactions of ABA with other hormones usually indicates that their antagonistic interactions are non-competitive.

It appears, therefore, that all 4 of these hormones act in concert to control the biosynthesis of dipeptidase through their action on nucleic acid metabolism. Cytokinins and GA act as stimulants; auxins act as stimulants or inhibitors, depending on concentration; and ABA acts as an inhibitor. Although they all appear to act primarily on nucleic acid metabolism, their primary site of action is probably not the same. Therefore, it appears that the biosynthesis of the dipeptidase in squash cotyledons is controlled by the relative influence of these hormones on nucleic metabolism. However, quantitive aspects of dipeptidase activity *in vivo* may also be controlled by an endogenous inhibitor(s).

EXPERIMENTAL

Seed germination. All experiments utilized seeds of *Cucurbita maxima* Duch. Chicago Warted Hubbard (squash). The seeds were surface sterilized in 0·5% sodium hypochlorite for 10 min; the nucellus was also partially removed in this process. The seeds were rinsed repeatedly in deionized-sterile H_2O until most of the nucellus tissue was removed. All manipulations were performed in a sterile transfer room and aseptic techniques were used.

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For the *de novo* synthesis study, twelve seeds and 12 ml H_2O were placed in a Petri dish and incubated at 27° for 4 days. The H_2O was removed and replaced with 12 ml H_2O containing 0·025 μCi (0·0148 mg) of uniformly labeled [^{14}C]leucine. They were incubated for an additional 24 hr; the cotyledons were removed, and the enzyme purified.

For the hormonal regulation studies, the seeds were separated into two equal groups. One group was used as intact seeds. Seeds in the other groups were transversely cut in half. The half seeds without embryo axis are referred to as distal-half cotyledons. Twelve intact seeds or 12 distal-half cotyledons were placed in one Petri dish. 12 or 6 ml of the solns. were used per Petri dish for intact seeds and distal-half cotyledons, respectively. The Petri dishes with enclosed seeds were then incubated in dark at 27° for 4 days. The cotyledons were removed and assayed for dipeptidase activity. Only the distal-half of the cotyledons from the intact seeds were used in the assay.

Purification procedure followed Ashton and Dahmen.[6] The dipeptidase purified by this method was homogeneous. Crude enzyme (fraction I) was prepared by blending four cotyledons in each ml 0·1 M K_3PO_4 buffer, pH 7·6, and centrifuging at 34000 *g* for 1 hr. A 40–80% acetone fraction of the crude enzyme preparations (acetone fraction, or fraction II) was dissolved in 0·05 M K_3PO_4 buffer, pH 7·6, and passed through Sephadex G50. The cloudy fraction (Sephadex fraction or fraction III) was placed on a DEAE column and eluted with a linear NaCl gradient from 0·0 to 0·35 M. The active fraction was called DEAE fraction or fraction IV.

Protein determination. Protein content were determined according to the method of Lowry *et al.*[24]

Test for radioactivity. The amount of [^{14}C]leucine incorporation into protein followed the method of Marcus and Feeley.[25] 0·5 ml of fraction I, II and III, and 5 ml from each tube in fraction IV was placed in a 12-ml conical centrifuge tube. Non-radioactive leucine (0·6 ml of 0·1 M) and 0·3 ml of 2% casein in 0·1 M K_3PO_4 buffer, pH 7·6 were added, followed by a mixing with a sufficient vol. of a 30% TCA soln to completely ppt. the protein. After standing 15 min the tubes were centrifuged for 5 min. The pellet from each tube was washed 2× with 4 ml of a cold 5% TCA solution. Each pellet was resuspended with 4 ml EtOH–Et_2O (1:1) and incubated in a water bath for 30 min at 30°. The resuspended soln was then centrifuged and its pellet was rewashed with 4 ml Et_2O. After centrifugation, the pellet was dried in a stream of air and dissolved in 0·5 ml HCOOH. 0·1 ml from each sample was placed in a vial, and 15 ml scintillation mixture (160 g naphthylene, 15 g 2,5-diphenyloxazole (PPO), 0·3 g of 1,4-*bis*-2-(5-phenyloxazole)-C_6H_6 (POPOP), 720 ml EtOH, 1140 ml toluene and 1140 ml *p*-dioxane) was added to each vial, and counted for 10 min.

Assay for enzyme activity was modified from the method described by Ashton and Dahmen.[6] Glycylglycine at 0·8 M, dissolved in 0·1 M K phosphate buffer, was used as the substrate. The reaction mixture contained 2 ml of the enzyme soln and 2 ml of the glycylglycine soln in a 25 ml conical flask. Each flask containing the reaction mixture was covered with a sheet of wax and incubated at 35° for 60 min in a shaking water bath. One ml was removed at zero time and after the incubation period; these were added to 1 ml of 36% formaldehyde solution, pH 7·0. After standing 10 min, the samples were centrifuged to remove the precipitated protein. The supernatant was titrated to pH 8·6 at 34° with 0·1 M KOH using an automatic titrator. The increased acidity was calculated as ml of 0·1 M KOH used to titrate the 60-min mixture minus the ml of titrant used to titrate the 0 time sample and expressed in units (1 unit = 1 ml of 0·1 M KOH/60 min). The hormones were added to an enzyme preparation from cotyledons of control plants grown in H_2O. The dipeptidase activity was not affected by the presence of any of the hormones.

The preparation of hormonal solution. Solns of BA and IAA were autoclaved for 15 min at 1 atm. for sterilization. Since GA was reported to lose biological activity when autoclaved,[26] and there was little information about the stability of ABA to heat, these two solns were sterilized by filtration through an autoclaved Millipore filter with pore size 0·45 μM.

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