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BIOSYNTHESIS OF CYANIDIN IN BUCKWHEAT HYPOCOTYLS

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Key Word Index—*Fagopyrum esculentum*; Polygonaceae; biosynthesis; aminooxyacetate; cyanidin; precursor complementation.

Abstract—Aminooxyacetate (AOA), an inhibitor of phenylalanine transamination and deamination *in vitro*, inhibits the light-induced formation of chlorogenic acid, leucoanthocyanin, rutin and anthocyanin (cyanidin glycosides) in buckwheat hypocotyls. Anthocyanin production is inhibited 87 ± 4%, when excised hypocotyls are incubated in 0.5 mM AOA in Petri dishes. AOA is also effective when taken up through the roots or sprayed onto seedlings. In the presence of biosynthetic precursors of cyanidin (L-phenylalanine, *trans*-cinnamic acid, *p*-coumaric acid, naringenin, eriodictyol, dihydrokaempferol. and dihydroquercetin) the inhibition of anthocyanin formation caused by AOA is completely or partially reversed. The general applicability of a complementation technique involving AOA or a similar inhibitor of phenylpropane synthesis is proposed to investigate the biosynthesis of natural products derived from cinnamic acid.

INTRODUCTION

Our understanding of biosynthetic pathways for natural products is based on 3 general approaches: the use of isotopically labelled precursors, the examination of enzyme systems in cell free extracts and the use of organisms with a block in the pathway under study [1, 2]. In the latter case, a biosynthetic pathway may be blocked genetically (auxotrophic mutant) or by metabolic inhibitors [3]. Tracer experiments established our basic knowledge of flavonoid biosynthesis in higher plants [4] and subsequent enzymatic studies greatly deepened and advanced our insight into the biochemistry and physiology of the formation of this class of natural compounds. Mutants blocked in anthocyanin synthesis have also increasingly been employed to elucidate the sequence of biosynthetic transformations at the C_{15}-stage of anthocyanidin synthesis with the aid of a complementation technique [6–12]. In the present communication, a complementation technique is introduced which utilizes a metabolic inhibitor to block anthocyanin synthesis. The inhibitor used is aminooxyacetic acid (AOA), which has previously been shown to be an effective inhibitor of anthocyanin (cyanidin glycosides [13]) synthesis in buckwheat hypocotyls [14, 15].

RESULTS

Light-induced anthocyanin synthesis in hypocotyls of etiolated buckwheat seedlings was reduced when the seedlings or excised hypocotyls were simultaneously treated with AOA (Fig. 1). The inhibition of anthocyanin formation as a function of AOA concentration was equally effective, when AOA was supplied to excised hypocotyls floating in a buffered solution in Petri-dishes or when seedlings were immersed with their roots or the cut ends of their hypocotyls in the inhibitor solution. 50% Inhibition was achieved at 5×10^{-5} to 10^{-4} M AOA concentrations. At 0.5 mM concentration AOA produced 87 ± 4% inhibition of anthocyanin formation in excised hypocotyls. AOA also effectively inhibited anthocyanin synthesis when sprayed onto the seedlings prior to the onset of illumination (Fig. 1); in this case higher concentrations had to be employed to achieve a comparable degree of inhibition. All subsequent investigations reported here were carried out with excised hypocotyls. When 1 mM AOA was added to the incubation buffer during mid-course of anthocyanin synthesis, inhibition was apparent within less than two hr (Fig. 2) indicating that AOA interacts directly with metabolic step(s) in the tissue rather than causing the disappearance of an enzyme required for anthocyanin formation. It has previously been shown that AOA hardly interferes with the light-induced increase

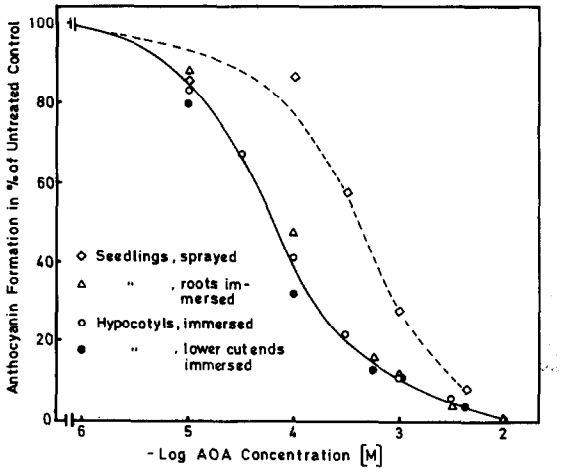

Fig. 1. Inhibition of anthocyanin synthesis in illuminated buckwheat hypocotyls as a function of AOA-concentration.

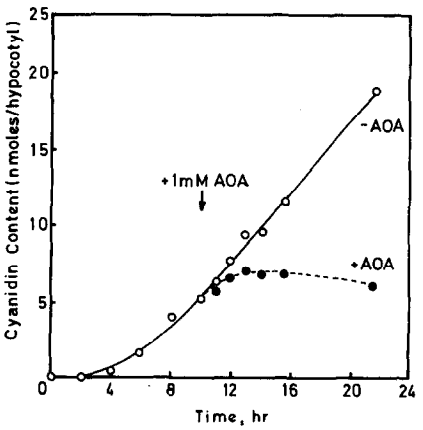

Fig. 2. Inhibition of light-induced anthocyanin synthesis in buckwheat hypocotyls after transfer (arrow) to 1 mM AOA.

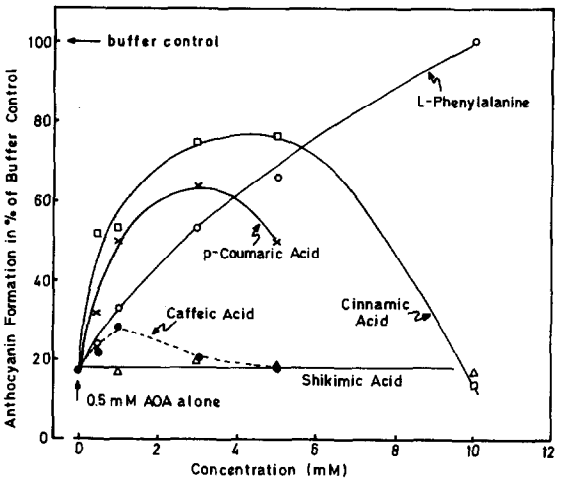

Fig. 4. Reversal of inhibition by AOA of anthocyanin synthesis in illuminated buckwheat hypocotyls as a function of shikimic acid and C_9-precursor concentration.

and subsequent decrease of phenylalanine ammonia-lyase (PAL) in buckwheat hypocotyls [15]. The inhibitory effect of AOA is not specific for anthocyanin synthesis; it rather inhibits the formation of all phenylpropanoid compounds, which have been identified in buckwheat hypocotyls [13] to a similar degree (Fig. 3). Chlorogenic acid, rutin and leucoanthocyanin are found already in the hypocotyls of dark-grown plants, but their accumulation is enhanced by light [13]. It is evident from Fig. 3 that this light-induced accumulation of phenylpropanoid derivatives is reduced by 0.5 mM AOA by 74–82%. Because anthocyanins are easily extracted and assayed we restricted the complementation studies described below to anthocyanins.

Since our earlier investigations had shown that AOA inhibits phenylalanine transamination and deamination competitively *in vitro* [14], we assumed that AOA *in vivo* blocks the formation of cinnamic acid, and possibly that of phenylalanine, thus depriving the hypocotyls of the substrate for the synthesis of the B-ring of flavonoids. Complementation of hypocotyls, in which anthocyanin synthesis was blocked by 0.5 mM AOA, with increasing concentrations of L-phenylalanine, *trans*-cinnamic acid, and *p*-coumaric acid abolished the inhibition partially, or, in the case of phenylalanine, completely (Fig. 4). Concentrations of the two acids higher than 4–5 mM caused loss of turgor of the hypocotyls and apparently damaged the tissue. Caffeic acid caused a marginal, but reproducible, enhancement of anthocyanin synthesis in the presence of 0.5 mM AOA (Fig. 4). Browning of the cut ends of the hypocotyls in the presence of caffeic acid indicated oxidation of the acid by the tissue. In the presence of 10 mM ascorbate browning was prevented, but anthocyanin synthesis was not increased. Application of 4-β-D-glucosyl caffeic acid, likewise, did not overcome the inhibition by AOA. AOA-mediated inhibition of anthocyanin formation was not overcome by various concentrations of shikimic (Fig. 4) or quinic acids, D-phenylalanine, L-tyrosine, D,L-α-phenylglycine, D,L-α-aminophenylbutyric acid, L-tryptophan, glycine, glycolic acid, L-alanine, L-glutamate, L-aspartate, L-phenyllactate, acetate, sucrose, *o*- or *m*-coumaric acids, and ferulic or sinapic acids. It is apparent from these results that reversal of inhibition by AOA of anthocyanin synthesis by L-phenylalanine, *trans*-cinnamic acid and *p*-coumaric acid is due to the function of these compounds as the precursors of the B-ring of the cyanidin molecule [4].

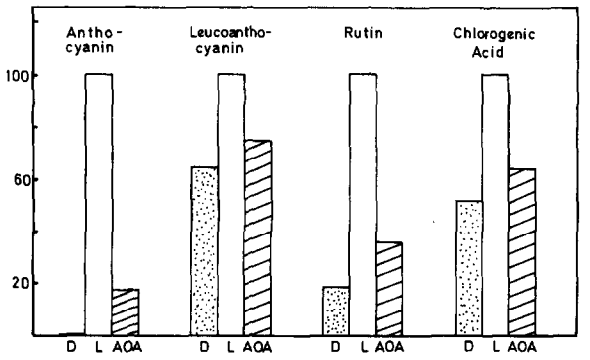

Fig. 3. Inhibition by 0.5 mM AOA of phenylpropanoid synthesis in illuminated buckwheat hypocotyls. Excised hypocotyls were incubated for 24 hr in the light or in darkness. Concentrations of phenylpropanoid compounds in illuminated hypocotyls are given as 100%. D = Dark controls; L = light control; AOA = light controls + 0.5 mM AOA.

Inhibiton by AOA of anthocyanin synthesis was also partially overcome by the simultaneous application of increasing concentrations fo the flavanones naringenin (5,7,4′-trihydroxyflavonone) and eriodictyol (5,7,3′,4′-tetrahydroxyflavanone), as well as the dihydroflavonols dihydrokaempferol (3,5,7,4′-tetrahydroxydihydroflavonol, aromadendrin) and dihydroquercetin (3,5,7,3′4′-pentahydroxyflavonol, taxifolin), as is evident from Fig. 5. All compounds were fed in a final concentration of 2% DMSO. Control experiments showed that DMSO in concentrations up to 5% did not interfere with anthocyanin formation under the conditions of our experiments. DMF could substitute for DMSO.

Naringin (naringenin-7-*O*-neohesperidoside), dihydrofisetin (3,7,3′,4′-tetrahydroxydihydroflavonol, fustin), apigenin (5,7,4′-trihydroxyflavone), kaempferol (3,5,7,4′-tetrahydroxyflavonol), quercetin (3,5,7,3′,4′-pentahy-

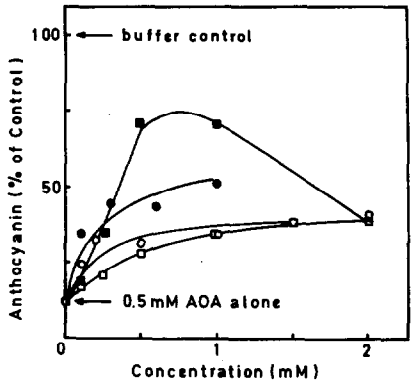

Fig. 5. Reversal of inhibition by AOA of anthocyanin synthesis in illuminated buckwheat hypocotyls as a function of C_{15}-precursor concentration. —■—■— naringenin; —□—□— eriocictyol; —●—●— dihydrokaempferol; —○—○— dihydroquercetin. All incubation solutions contained DMSO in a final concentration of 2%.

droxyflavonol) and the 4'-methylated derivative of eriodictyol (hesperetin) were ineffective, while the 3'-methylated derivative of eriodictyol (homoeriodictyol) showed marginal activity. Complete reversal of AOA-mediated inhibition was also obtained with phenylpyruvate, *p*-OH-phenylpyruvate, pyruvate, acetone, and α-ketoglutarate. The action of these compounds is, however, not related to their possible function as intermediates in anthocyanin synthesis. They rather bind AOA by oxime formation and thus reduce the active concentration of the inhibitor. (When AOA was added to a pyruvate solution in equimolar amounts, for example, the *A* at 546 nm of the 2,4-dinitrophenylhydrazone, which was formed upon addition of 2,4-dinitrophenylhydrazine to the pyruvate solution, was reduced by 50%.) EtOH (but not MeOH, PrOH, *n*-BuOH, *iso*-BuOH and glycerol) also effectively reversed inhibition of anthocyanin formation by AOA.

When 2,4-dinitrophenol was used as a metabolic inhibitor to block anthocyanin formation [16, 17], the inhibition could be reversed by naringenin, but not by L-phenylalanine, while inhibition by AOA was reversed by both compounds (Table 1). The metabolic step inhibited by 2,4-dinitrophenol is thus most likely the energy requiring formation of the hydroxycinnamoyl-CoA esters and malonyl-CoA, which are the substrates for flavanone synthase [23–25].

Table 1. Effect of L-phenylalanine and naringenin on inhibition of anthocyanin synthesis by AOA and 2,4-dinitrophenol in illuminated buckwheat hypocotyls

Incubation mixture	Anthocyanin (% of control)
Buffer alone	100
5×10^{-5} M 2,4-DNP	33
5×10^{-5} M 2,4-DNP + 10 mM L-phenylalanine	37
5×10^{-5} M 2,4-DNP + 0.5 mM naringenin	67
5×10^{-4} M AOA	21
5×10^{-4} M AOA + 10 mM L-phenylalanine	85
5×10^{-4} M AOA + 0.5 mM naringenin	71

All incubation solutions contained DMSO in a final concentration of 2%.

DISCUSSION

AOA has long been recognized for its general reactivity towards pyridoxal phosphate-dependent enzymes such as amino acid transaminases and decarboxylases, of microbial and animal origin, and has been used *in vivo* in animal tissues to differentiate between metabolic pathways of amino acids (see [18] for references). The compound has, to my knowledge, only sporadically been used in studies of higher plant pyridoxal phosphate-dependent enzymes and was patented as an agent to control plant growth [19]. We have previously shown that AOA inhibits phenylalanine deamination and transamination *in vitro* [14] and phenylpropanoid biosynthesis *in vivo* [14, 15]. Inhibition of phenylpropanoid synthesis by AOA cannot be considered specific, because AOA inhibits growth as well [15], and inhibition of growth cannot be reversed by the simultaneous application of L-phenylalanine. Reversal of the AOA-mediated inhibition of anthocyanin synthesis by intermediates of phenylpropanoid metabolism in general (Fig. 3) and flavonoid (cyanidin) synthesis in particular (Fig. 4) shows, however, that AOA does not irreversibly abolish the tissue's potential to produce anthocyanins.

It is generally accepted that ring B of the flavonoid skeleton is derived from L-phenylalanine via cinnamic and *p*-coumaric acid [4, 5]. The data presented in Fig. 3 agree with this scheme. The fact that complete reversion of AOA-mediated inhibition of anthocyanin synthesis was achieved only with L-phenylalanine, but not cinnamic and *p*-coumaric acid, is probably primarily due to the fact that the tissue does not tolerate high concentrations of the cinnamic acids, while it does tolerate 10 mM phenylalanine. On the other hand, these results may be due to the fact that the cinnamic acids produced endogenously are not in equilibrium with the exogenously added acids. Indications for such a situation were obtained recently in *in vitro* [20] and *in vivo* [21] studies of phenylpropanoid metabolism. The inefficiency of caffeic acid in the reversion of the inhibition of anthocyanin synthesis by AOA is of interest in relation to the question at which stage of flavonoid synthesis the oxygenation pattern of ring B is determined ('cinnamic acid starter hypothesis' [22] vs 'substitution hypothesis' [5]). If, in the case of buckwheat, the cinnamic acid starter hypothesis is valid, caffeic acid should reverse the inhibition, since cyanidin has the same oxygenation pattern in ring B as caffeic acid. The low efficiency of caffeic acid in the complementation experiments (Fig. 3) may indicate either that caffeic acid is not the precursor of cyanidin or that the exogenously supplied compound does not have access to the site of its activation by hydroxycinnamoyl-CoA ligase and the subsequent conversion by flavanone synthase to the corresponding flavanone (eriodictyol). Since caffeic acid is susceptible to attack by phenoloxidases, as indicated by the browning of the cut ends of the hypocotyls in the presence of the acid, an attempt was made to protect caffeic acid from oxidation during uptake by supplying it either along with ascorbate as a reducing agent or as its 4-β-D-glucosyl derivative. None of these attempts was successful, however. As discussed below, naringenin, having the substitution pattern of *p*-coumaric acid in its B-ring, effectively reversed the AOA-mediated inhibition of anthocyanin synthesis. It must, therefore be concluded that *m*-

hydroxylation of the B-ring can occur at the C_{15}-stage of flavonoid biosynthesis. While studies on the substrate specificity of flavanone synthetase *in vitro* seemed first to indicate that the enzyme utilized exclusively *p*-coumaroyl-CoA [23], it was later found that caffeoyl-CoA may also be utilized [24, 25], so that naringenin, as well as eriodictyol, have to be considered natural products of the synthase reaction. Reversal of AOA-inhibition by 4′-hydroxylated (naringenin, dihydrokaempferol), as well as 3′, 4′-hydroxylated (eriodictyol, dihydroquercetin) flavanones and dihydroflavonols, respectively, (Fig. 4), indicates clearly that B-ring *m*-hydroxylation can occur at the C_{15}-stage, which is in agreement with tracer experiments [26–28], enzymatic studies [28–30] and complementation experiments with mutants [11]. The complementation technique involving AOA presented here has also provided additional and independent evidence for the participation of dihydroflavonols in cyanidin biosynthesis.

The scheme for cyanidin biosynthesis in buckwheat hypocotyls evolving from the present results is thus in close agreement with that proposed earlier by Grisebach and coworkers [26–28]:

Phenylalanine → Cinnamic acid → *p*-Coumaric acid → Naringenin → [Eriodictyol / Dihydrokaempferol] → Dihydroquercetin → Cyanidin.

The relative efficiency of a compound in the reversal of inhibition by AOA of anthocyanin synthesis (Figs. 3 and 4) probably gives little indication of its efficiency as a precursor, since differences in uptake, transport within the tissue and cells, pool sizes and utilization in other reactions have to be taken into account [1]. The relatively low efficiency of the C_{15}-precursors used in the present investigation as compared to that of the C_9-precursors (Figs. 3 and 4) may be due to such differences, especially compartmentation of anthocyanidin biosynthesis at the cellular level [28]. Attempts to increase anthocyanin synthesis in the complementation studies with the aid of membrane permeabilizing agents, such as organic solvents or detergents, did not meet with success. Mechanical treatment of the hypocotyls (incisions with a razor blade, gentle rubbing with aluminum oxide), likewise, rather decreased than increased the efficiency of the precursors. It was also noticed that anthocyanin distribution in the complemented hypocotyls tended to be in patches as compared to a homogeneous distribution in the controls. Such an irregular pigmentation was also observed in complementation experiments with flower petals [8].

While the present study has essentially confirmed results obtained with other methods, it serves its main purpose to point out the potential of the complementation technique presented here in the investigation of the biosynthesis of flavonoids and other phenylpropanoid substances with a more complex oxygenation and methoxylation pattern. Experiments on these lines, involving also the more specific inhibitor of phenylpropanoid metabolism, α-aminooxy-β-phenylpropionic acid [31], are in progress.

EXPERIMENTAL

Aminooxyacetic acid semihydrochloride was provided by Sigma, St. Louis MO. Homoeriodictyol was isolated from the drug Yerba santa (*Eriodictyon californicum* Greene) obtained from Interdrogas, Cologne, according to a procedure made available by Prof. H. Wagner, Munich, who also provided a sample of dihydrokaempferol. Eriodictyol was obtained by demethylation [32] of hesperetin. 4-β-D-glucosyl caffeic acid was synthesized according to ref. [33]. Caffeic acid was recrystallized twice from H_2O and found to be chromatographically pure (PC; 4% HCO_2H). All other compounds were commericially available from the usual sources.

Experimental procedures. References to the plant material, cultivation of seedlings, incubation of hypocotyls, as well as the extraction and quantitative estimation of phenylpropanoid substances from the hypocotyls are as in refs. [13, 14]. Unless stated otherwise, 20 excised hypocotyls from 6-day-old etiolated buckwheat seedlings were incubated in Petri dishes under constant illumination for 24 hr. The incubation soln consisted of 10 ml 0.01 M Pi buffer, pH 5.5, with appropriate additions as indicated in the text.

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