Isoxaben Inhibits the Synthesis of Acid Insoluble Cell Wall Materials In Arabidopsis thaliana

Dale R. Heim, John R. Skomp, Edward E. Tschabold, and Ignacio M. Larrinua*
DowElanco, P. O. Box 708 Greenfield, Indiana 46140

ABSTRACT

The effect of the herbicide isoxaben on the incorporation of radiolabeled glucose, leucine, uracil, and acetate into acid insoluble cell wall material, protein, nucleic acids, and fatty acids, respectively, was measured. Dichlobenil, cycloheximide, actinomycin D, and cerulenin, inhibitors of the incorporation of these precursors into these macromolecular components, functioned as expected, providing positive controls. The incorporation of radiolabeled glucose into an acid insoluble cell wall fraction was severely inhibited by isoxaben at nanomolar concentrations. Amitrole, fluridone, ethalfluralin, and chlorsulfuron, as well as cycloheximide, actinomycin D, and cerulenin did not inhibit incorporation of glucose into this fraction, ruling out a general nonspecific effect of herbicides on glucose incorporation. The evidence thus suggests that isoxaben is an extremely powerful and specific inhibitor of cell wall biosynthesis.

METHODS AND MATERIALS

Plant Material and Growth Conditions

Seed from Arabidopsis thaliana var. Columbia were washed and sterilized as described in Somerville and Ogren (9). After the final wash, seed that had settled to the bottom of a 14 mL plastic Sarstedt tube were drawn up into 55 μL aliquots using a micropipetter (Pipetman) whose tip had been excised with scissors to increase its diameter. The seeds were dispensed into sterile, 18 x 150 mm, borosilicate glass culture tubes containing 5 mL of the sterile Arabidopsis growth media of Somerville and Ogren (9) except that 0.5% glucose was used instead of sucrose. The tubes, containing about 200 seeds each, were incubated in the dark for 4 d at 27°C with continuous rotation on a rollodrum (New Brunswick Instrument Co. TC-7; setting No.6).

The germinating seedlings were used after 4 d as the starting material for all of the experiments and will be subsequently denoted as the A. thaliana plants.

Chemicals

All radiolabeled materials were obtained from NEN Research Products. DMSP was from EM Products. Leucine, uracil, and sodium acetate were from Sigma. Isoxaben, 89% pure by weight, and fluridone were the kind gifts of Dr. Ken Burow and Dr. Riaz Abdulla (Lilly Research Laboratories), respectively. Ethalfluralin, 96% pure, was obtained from the Herbicide Screening Group (Lilly Research Laboratories). Dichlobenil and chlorsulfuron were purchased from Chem Service, Inc., and amitrole was from Alfa Products.

Incorporation of 14C Glucose

Arabidopsis plants were washed three times with 10 mL of growth media without glucose before resuspending in 3 mL of the treatment solution. This solution consisted of growth media with 1.0 μCi/mL [14C]glucose (3.7 mCi/mmol), and the appropriate inhibitor concentration with a final DMSO content of 0.025%. Controls were treated as above but no herbicide was added. The plants were then incubated for 1 h in the dark at 27°C in the rollodrum as above (incubation
time was varied for the time course experiment). After the treatment, plants were washed three times with glucose-free growth media and excess media was removed by blotting onto paper towels. Then, 10 mL of an acetic acid:nitric acid:water solution as described by Updegraff (12) was added and the samples were incubated for 2 h in a boiling water bath.

Acid insoluble cell wall material was collected onto preweighed glass microfiber filters (Whatman GF/C; 2.4 cm) by vacuum filtration, washing each sample with 30 mL of deionized water. Filters were freeze dried for at least 2 h before taking dry weights. Filters were then combusted in a biological oxidizer-OX400 (R. J. Harvey Instrument Corp.) and counts were collected into Carbon 14 Cocktail (R. W. Rawlings Instrument Co.) for dpm determinations. All treatments consisted of at least five repetitions within each experiment.

**Incorporation of \(^{14}\text{C} \) Leucine**

Labeled leucine incorporation into protein was determined using a modification of the procedure of Lefebvre et al. (7). *A. thaliana* plants were drained of growth media prior to the addition of 3 mL of the treatment solution. The treatment solution consisted of growth media with 0.5% glucose, 10\(^{-5}\) M leucine, 0.1 \(\mu\text{Ci}/\text{mL} \) \(^{14}\text{C}\)-leucine (318 mCi/mmol), and 0.025% DMSO with or without the addition of the herbicide. The cycloheximide concentration was 1 \(\mu\text{M}\). The plants were incubated for 1 h (varied for time course) in the dark at 27°C in the rorodrum as above. After the treatment period, the plants were washed four times with 6 mL of growth media before grinding in 3 mL cold 10% TCA in a hand held glass to glass tissue grinder. The tissue grinder was washed with 12 mL 10% cold TCA before the 15 mL were filtered onto two separate, preweighed glass filters (Whatman GF/C; 2.4 cm). The tissue grinder was rinsed with 15 mL cold 10% TCA and this was also filtered through the two filters. Ten mL additional TCA was used to wash each filter followed by a 10 mL cold 80% ethanol wash. Filters were freeze dried overnight, weighed, and counted as above. Data points are the result of three repetitions per treatment.

**Incorporation of \(^{14}\text{C} \) Uracil**

Labeled uracil incorporation into nucleic acids was determined by a modification of the procedure of Lefebvre et al. (7). *A. thaliana* plants were treated as above except that the treatment solution consisted of growth media with 0.5% glucose, 10\(^{-5}\) M uracil, 0.1 \(\mu\text{Ci}/\text{mL} \) \(^{14}\text{C}\)-uracil (54 mCi/mmol), and 0.025% DMSO with or without the treatment compound. The actinomycin D concentration was 100 \(\mu\text{g}/\text{mL} \). TCA insoluble material was collected onto preweighed glass filters and dpm per mg determined as above.

**Incorporation of \(^{14}\text{C} \) Acetate**

Labeled acetate incorporation into lipids was determined by a modification of the procedure of Lefebvre et al. (7). *A. thaliana* plants were treated as above except the treatment solution consisted of growth media with 0.5% glucose, 10\(^{-5}\) M sodium acetate, 0.1 \(\mu\text{Ci}/\text{mL} \) \(^{14}\text{C}\)-acetic acid (56.6 mCi/mmol), and 0.025% DMSO with or without the treatment compound. The cerulien concentration was 50 \(\mu\text{g}/\text{mL} \). Plants were washed four times with 6 mL of growth media, dried on paper towels as completely as possible, and the samples weighed. These were frozen at \(-80^\circ\text{C}\) for at least 2 h, thawed in 6 mL chloroform:methanol (2:1 ratio), and incubated overnight at room temperature. Each sample was filtered through a glass filter and the insoluble material washed with 3 mL of the chloroform:methanol solution. The liquid from the solvent extraction was dried to completion under a constant stream of air. The remaining material was resuspended in 2 mL of hexane and 2 mL of deionized water and mixed by shaking. This two-phase system was used to partition water soluble counts away from those incorporated into the lipid fraction. The hexane phase was then added to 10 mL of PCS scintillation fluid (Amersham Corp.) for dpm determination. Samples are expressed as dpm per 100 mg wet weight.

**RESULTS**

**Incorporation of Radiolabeled Precursors is Linear With Time**

To validate the test system, the incorporation of radiolabeled precursors into high mol wt compounds over the time course of the experiment needed to be linear. As can be seen from Figure 2, incorporation into macromolecular components of all four radiolabeled precursors—acetate, glucose, leucine, and uracil—was linear for at least 2 h after addition of the compounds. Therefore all of the experiments were terminated after 1 h. An additional glucose inhibition concentration curve was conducted for 2 h to see if inhibition by isoxaben could be increased any further.

**Isoxaben Does Not Inhibit Incorporation of Leucine, Uracil, or Acetate**

As can be seen from Table I isoxaben at a concentration of 100 \(\mu\text{M}\) failed to significantly inhibit the incorporation of leucine, uracil, or acetate into high mol wt material. To show that these processes were sensitive to inhibition at the time they were tested, known inhibitors were included as positive controls. Table I shows that, as expected, cycloheximide reduced leucine incorporation into TCA insoluble material by
ISOXABEN BLOCKS INCORPORATION OF GLUCOSE INTO CELL WALL MATERIAL

Figure 2. Incorporation of radiolabeled glucose, leucine, uracil, and acetate into higher mol wt components in Arabidopsis as a function of time.

more than 80%. Actinomycin D had a similarly profound effect on the incorporation of uracil. Cerulenin, an inhibitor of fatty acid elongation, had a weaker effect on the incorporation of acetate into fatty acids (about 30%). This may be due to the compound not being readily taken up. Published inhibition experiments with cerulenin allow for a lengthy preincubation with the inhibitor (8). Nevertheless, isoxaben actually stimulated the incorporation of acetate into fatty acids by 14%.

We can conclude that isoxaben does not primarily inhibit protein, nucleic acids, or fatty acid synthesis at physiologically meaningful concentrations.

Isoxaben Inhibits the Incorporation of Glucose into Acid Insoluble Material

The uptake of glucose by Arabidopsis seedlings was not inhibited by isoxaben, uptake of glucose being 95% of control (DR Heim, JR Skomp, C Waldron, IM Larrinua, unpublished data). Isoxaben did inhibit the incorporation of radiolabeled glucose into an acid insoluble cell wall fraction, usually assumed to be cellulose (12) (Fig. 3). This inhibition occurred at similar isoxaben concentrations as the in vivo inhibition (3), strengthening the conclusion that it is physiologically relevant. Maximal inhibition of glucose incorporation, about 80%, occurred at approximately 100 nM (Fig. 3), with a no effect level of approximately 1 nM. This, presumably, is the primary site of activity for isoxaben.

The maximal level of inhibition did not increase with incubation time (Fig. 3) nor with concentrations of isoxaben as high as 10,000 nM (Fig. 4). The possible relevance of this isoxaben insensitive glucose incorporation will be discussed later.

Inhibition of Glucose Incorporation Is Not a Generalized Effect of Inhibitors

It was possible that the inhibition of glucose incorporation, although specific to that precursor, was due to a secondary, generalized response by the plant to herbicidal injury. For example, cessation of cell growth, might lead to a shutdown of cell wall biosynthesis. Therefore, we tested a number of herbicides at concentrations 10-fold higher than the lethal dose to A. thaliana (data not shown) to see if any mimicked the effect of isoxaben on glucose incorporation. The compounds tested were amitrole, an herbicide whose mode of action is unknown, fluridone, a carotenoid biosynthesis inhibitor (1), ethalfluralin, a microtubule inhibitor (4), and chlorosulfuron, an inhibitor of branched amino acid biosynthesis (6). Ethalfluralin was included because microtubules are thought to help orient the growth of the cell wall. Chlorosulfuron was included because one of its earliest effects is to stop cell division. Either of these two effects could, in theory, have caused rapid cessation of cell wall biosynthesis. Fluridone was added to test for more distant effects.

As shown in Figure 5 none of these herbicides had any significant effect on the incorporation of glucose into the acid insoluble cell wall material. The effect of cycloheximide, actinomycin D, and cerulenin on the incorporation of radioactive glucose into this fraction was also measured (data not shown) and none of these inhibitors significantly inhibited this process.

Table I. Effect of Isoxaben and Known Inhibitors on Protein, Nucleic Acid, and Fatty Acid Synthesis

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpm</td>
<td>%</td>
</tr>
<tr>
<td>1. Control</td>
<td>12283 ± 1124</td>
<td>100</td>
</tr>
<tr>
<td>2. Isoxaben</td>
<td>12042 ± 170</td>
<td>98</td>
</tr>
<tr>
<td>3. Cycloheximide</td>
<td>2580 ± 133</td>
<td>21</td>
</tr>
<tr>
<td>4. Control</td>
<td>1065 ± 150</td>
<td>100</td>
</tr>
<tr>
<td>5. Isoxaben</td>
<td>1116 ± 86</td>
<td>105</td>
</tr>
<tr>
<td>6. Actinomycin D</td>
<td>170 ± 7</td>
<td>16</td>
</tr>
<tr>
<td>7. Control</td>
<td>1756 ± 107</td>
<td>100</td>
</tr>
<tr>
<td>8. Isoxaben</td>
<td>2000 ± 80</td>
<td>114</td>
</tr>
<tr>
<td>9. Cerulenin</td>
<td>1303 ± 45</td>
<td>74</td>
</tr>
</tbody>
</table>

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Thus, it can be concluded that the effect of isoxaben on glucose incorporation is not a generalized phenomenon induced by injury, and strengthens the possibility that it is the primary site of action.

**Dichlobenil Has a Similar Effect to Isoxaben**

Dichlobenil is an herbicide that has been shown to inhibit glucose incorporation into cellulose (2). Furthermore, the suggestion had previously been made that the mode of action of isoxaben and dichlobenil was similar (7). Thus, it was of interest to see if dichlobenil was active in this system. As shown in Figure 4, dichlobenil inhibits the incorporation of glucose into acid insoluble material in a concentration-dependent manner. The main difference between the two herbicides is that in this test isoxaben is 40 times more active than dichlobenil.

**DISCUSSION**

Isoxaben is a new and powerful herbicide whose mode of action is not yet completely understood. A two-pronged approach to the understanding of the mode of action of this herbicide is being used. We, we have isolated mutants resistant to isoxaben (3, 3a). These mutants are now being used to further characterize the mode of action of isoxaben (DR Heim, JR Skomp, C Waldron, IM Larrinua, unpublished data). In this communication, the biochemical characterization of the effects of this herbicide is begun.

Lefebvre et al. (7) studied the effects of isoxaben on the morphology and metabolism of *Acer* and *Glycine* tissue culture cells. In *Acer* they reported a 55% decrease in radiolabeled leucine incorporation into TCA precipitable material, and a 25% decrease in glucose incorporation into a crude cell wall fraction at an herbicide concentration of 100 μM. This and the similarity of the morphological effects of isoxaben and dichlobenil on *Glycine* tissue culture cells led these investigators to postulate that protein synthesis and cell wall biosynthesis might be the primary sites of action of isoxaben (7).

The above results did not discriminate between protein synthesis and cell wall biosynthesis as the primary mode of action of isoxaben. Furthermore, the concentrations needed to see these effects raised the question of whether they were due to a primary or a secondary site of action.

We have measured the effects of isoxaben on the incorporation of radiolabeled precursors using hydroponically grown *Arabidopsis* seedlings. Incorporation of radiolabeled precursors into these seedlings is linear for at least 2 h, enough time to look at the primary effects of an herbicide.

Isoxaben does not affect the incorporation of radiolabeled acetate, leucine, or uracil into these seedlings at concentrations 20 times higher than the *I*<sub>0</sub> *in vivo*. This is in spite of...
the fact that known inhibitors of the reactions tested—cyclohexamide, actinomycin D, and cerulenin—were active in our system. Thus, it is possible to confirm that isoxaben does not inhibit incorporation of acetate or uracil, but not possible to confirm any effect on protein biosynthesis. The inhibition seen by Lefebvre et al. (7) may be a secondary effect due to the concentrations of the herbicide used, or may represent a real difference between the experimental systems used.

We are able to confirm and extend their observation that isoxaben, like dichlobenil affects cell wall biosynthesis. In our system, inhibition of glucose incorporation into an acid insoluble material occurs at extremely low herbicide concentrations, having an $I_{50}$ of 10 nm, which is within a factor of 2 of the in vivo $I_{50}$ (3). This inhibition peaks at 80% of the control incorporation at approximately 30 to 100 nm. This increased sensitivity and increased inhibition over that seen by Lefebvre et al. (7) is due to several factors: whole plants can be 50-fold more sensitive than tissue cultures of the same species (3); different species were used in these studies, and Lefebvre et al. (7) assayed a crude organic solvent washed cell wall preparation, instead of an acid insoluble cell wall fraction, a subcomponent of the above composed predominantly of cellulose (12).

This data clearly demonstrates that isoxaben is a powerful and specific inhibitor of cell wall biosynthesis. Whether it inhibits cellulose biosynthesis specifically or some other effect in the plant that is tightly linked to cell wall biosynthesis is under investigation.

Complete inhibition of glucose incorporation was not possible. The two most likely explanations are that this fraction is contaminated with polymers from pathways insensitive to isoxaben or that we are looking at residual activity of the inhibited pathway. It is also possible that we are looking at a minor secondary pathway producing the same end product but insensitive to the herbicide. This would have obvious implications in terms of herbicide selectivity.

It was possible that the inhibition of glucose incorporation seen was a generalized effect of herbicidal activity per se or one of the secondary effects common to a large number of herbicidal modes of action. If this was true, inhibitors with unrelated modes of action should show a similar effect. To eliminate this possibility, several inhibitors with different or unknown modes of action were tested to see if they would have the same effect as isoxaben. Neither cycloxeximide, actinomycin D, cerulenin, amitrole, fluridone, ethalfluralin, nor chloronsulfuron inhibited glucose incorporation into acid insoluble material. These observations strengthen the conclusion that this effect is a specific and primary effect.

Finally, dichlobenil was tested in this system, since this is a known cellulose inhibitor (2). Dichlobenil as expected, was able to inhibit glucose incorporation into the acid insoluble material, but at concentrations approximately 40-fold higher than isoxaben. This is in agreement with field data, where isoxaben is much more active than dichlobenil (11).

These observations point to the cell wall in general, and to the acid insoluble cell wall fraction in particular, as the site of action of isoxaben, but many questions remain unanswered. A careful fractionation of the cell wall needs to be done to see if this is the only fraction affected, and the identity of the affected fraction should be confirmed by compositional and linkage analysis.

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LITERATURE CITED