Journal of Plant Physiology **166** (2009) 1343—1349

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JOURNAL OF PLANT PHYSIOLOGY

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Benzaldehyde dehydrogenase from chitosan-treated *Sorbus aucuparia* cell cultures

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Received 19 December 2008; received in revised form 10 February 2009; accepted 7 March 2009

KEYWORDS
Benzaldehyde dehydrogenase;
Benzoic acid biosynthesis;
Biphenyl metabolism;
Phytoalexin;
Sorbus aucuparia

Summary

Cell cultures of *Sorbus aucuparia* respond to the addition of chitosan with the accumulation of the biphenyl phytoalexin aucuparin. The carbon skeleton of this inducible defense compound is formed by biphenyl synthase (BIS) from benzoyl-CoA and three molecules of malonyl-CoA. The formation of benzoyl-CoA proceeds via benzaldehyde as an intermediate. Benzaldehyde dehydrogenase (BD), which converts benzaldehyde into benzoic acid, was detected in cell-free extracts from *S. aucuparia* cell cultures. BD and BIS were induced by chitosan treatment. The preferred substrate for BD was benzaldehyde ($K_m = 49\,\mu M$). Cinnamaldehyde and various hydroxybenzaldehydes were relatively poor substrates. BD activity was strictly dependent on the presence of NAD^+ as a cofactor ($K_m = 67\,\mu M$).

Introduction

Sorbus aucuparia (mountain ash) is a species of the rosaceous subfamily Maloideae, which also includes the economically important members *Malus domestica* (apple) and *Pyrus communis* (pear). The phytoalexins of the Maloideae are biphenyls and dibenzofurans, which accumulate as inducible defense compounds in the sapwood (Kokubun and Harborne, 1995). An exception is the leaves of *S. aucuparia*, which form the biphenyl aucuparin in response to biotic and abiotic stress (Kokubun and Harborne, 1994). Aucuparin also accumulates in *S. aucuparia* cell cultures upon treatment with yeast extract (Liu et al., 2004). The carbon skeleton of biphenyls is formed by a type III polyketide synthase, biphenyl synthase (BIS; EC

Abbreviations: BD, benzaldehyde dehydrogenase; BIS, biphenyl synthase; DTT, dithiothreitol; MSTFA, *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide.

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doi:10.1016/j.jplph.2009.03.003

2.3.1.177; Liu et al., 2007). This enzyme catalyzes the iterative condensation of benzoyl-CoA with three molecules of malonyl-CoA to yield a linear tetraketide intermediate that is subsequently cyclized into 3,5-dihydroxybiphenyl under the loss of the terminal carboxyl group.

The biosynthesis of the starter substrate, benzoyl-CoA, is poorly understood. Benzoate:CoA ligase catalyzing the thioesterification of benzoic acid by coenzyme A has been detected in *Clarkia breweri* and *Hypericum androsaemum* (Abd El-Mawla and Beerhues, 2002; Beuerle and Pichersky, 2002). Benzoic acid is derived from cinnamic acid, and multiple pathways have been reported for this reaction. The shortening of the cinnamic acid side chain by a C_2 unit uses either a CoA-dependent and β-oxidative mechanism analogous to the β-oxidation of fatty acids, or a CoA-independent and non-β-oxidative route involving benzaldehyde as an intermediate (Wildermuth, 2006; Sircar and Mitra, 2008). A combination of these two mechanisms, CoA-dependent and non-β-oxidative, has been detected in *H. androsaemum* cell cultures (Abd El-Mawla et al., 2002) and in transgenic hairy root cultures of *Datura stramonium* expressing a bacterial gene encoding hydroxycinnamoyl-CoA hydratase/lyase (Mitra et al., 2002). To date, the multiple benzoic acid biosynthetic pathways have not been well defined and none of the genes involved have been cloned from plants (Wildermuth, 2006).

Here we report detection of benzaldehyde dehydrogenase (BD; EC 1.2.1.28) in chitosan-treated cell cultures of *S. aucuparia*. The properties of BD, which catalyzes the last step of benzoic acid formation (Figure 1), were studied in desalted cell-free extracts. The detection and characterization of this enzyme provide the first insight into benzoic acid metabolism in the economically valuable taxon Maloideae.

Materials and methods

Chemicals, plant material, elicitor treatment

Chemicals for enzyme assays were purchased from Sigma Aldrich (Taufkirchen, Germany). Cell cultures of *Sorbus aucuparia* were grown as described by Liu et al. (2004). Five-day-old cell cultures from the linear growth phase were treated with chitosan at a final concentration of 25 mg/L. The chitosan stock solution was prepared according to Pitta-Alvarez and Giulietti (1999).

Enzyme extraction

Soluble proteins were extracted from elicitor-treated cell cultures at 0–4 °C. Cells (5 g) were collected by filtration, mixed with 10% (w/w) Polyclar AT (Serva, Heidelberg, Germany) and homogenized in 4 mL of 100 mM HEPES buffer pH 8.0 containing 10 mM dithiothreitol (DTT). The homogenate was centrifuged at 9000 rpm for 25 min at 4 °C. An aliquot of the supernatant (2.5 mL) was freed from the low molecular mass substances by gel filtration through a PD_{10} column (GE Healthcare, Freiburg, Germany) equilibrated with 200 mM Tris–HCl buffer pH 7.5. The cell-free extracts used to measure BIS activity were prepared as described by Liu et al. (2004), except that an ammonium sulphate precipitation was omitted. Protein concentrations were determined according to Bradford (1976) using bovine serum albumin (BSA) as a protein standard.

Benzaldehyde dehydrogenase (BD) and biphenyl synthase (BIS) assays

BD activity was assayed essentially as described by Sircar and Mitra (2008). The standard assay mixture consisted of 0.5 mM benzaldehyde, 1 mM NAD^+, and 130 μg protein. The final assay volume was adjusted to 200 μL with 200 mM Tris–HCl buffer pH 9.5. The reaction mixture was incubated at 40 °C for 30 min. Control assays contained boiled protein extract. The reaction was stopped by adding an equal volume of methanol:acetic acid = 9:1. After rigorous shaking, the sample was centrifuged at 10,000*g* for 10 min and the resulting supernatant was analyzed by HPLC. Three independent experiments were performed, and average values were calculated. To examine the pH optimum, 200 mM potassium phosphate buffer ranging from pH 5.5 to 6.5 and 200 mM Tris–HCl buffer

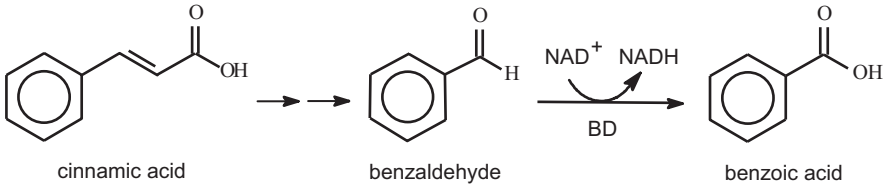

Figure 1. Benzaldehyde dehydrogenase (BD)-catalyzed formation of benzoic acid from benzaldehyde, itself derived from cinnamic acid.