4-Hydroxybenzoic acid biosynthesis in hairy root cultures of *Daucus carota* L.

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Introduction:

Hydroxybenzoic acid has received considerable attention because of its application in food, medicine, cosmetics, and polymer industries. The antimicrobial, anticancerous and anti-HIV activity of this hydroxybenzoate has also been well demonstrated. Despite the simple structure of 4-hydroxybenzoic acid and its widespread occurrence in plants, the enzymatic steps of 4-hydroxybenzoic acid biosynthesis is not well defined [1]. Mostly, plant hydroxybenzoates are likely to be derived from phenylalanine *via* general phenylpropanoid pathway involving 4-coumaric acid [2].

4-Hydroxybenzoic acid biosynthesis from L-phenylalanine requires shortening of the propyl- side chain by two carbons (C_2) , which can occur via a *β*-oxidative pathway or a non-*β*-oxidative pathway or combination of both the pathway [3]. Non-*β*-oxidative pathway proceeds through formation of 4-hydroxybenzaldehyde, a key intermediate.

In the present study, the enzyme system involved in non-*β*-oxidative pathway have been characterized in details using hairy root cultures of *Daucus carota* as experimental system.

Key Findings:

In hairy roots of *D. carota* 4-hydroxybenzoic acid biosynthesis proceeds *via CoA-independent* and non-*β*-oxidative pathway involving early intermediates of general phenylpropanoid pathway. The key enzymes of 4-hydroxybenzoic acid pathway are 4-hydroxybenzaldehyde synthase (HBS) and 4-hydroxybenzaldehyde dehydrogenase (HBD).

HBS catalyzes the C2-side chain cleavage of 4-coumaric acid leading to the formation of 4-hydroxybenzaldehyde, a key intermediate. This reaction was found to be thiol-reagent dependent.

HBD catalyzes NAD^+ dependent dehydrogenation of 4-hydroxybenzaldehyde into 4-hydroxybenzoic acid.

HBS and HBD enzymes were biochemically characterized using cell-free extract from elicited hairy roots.

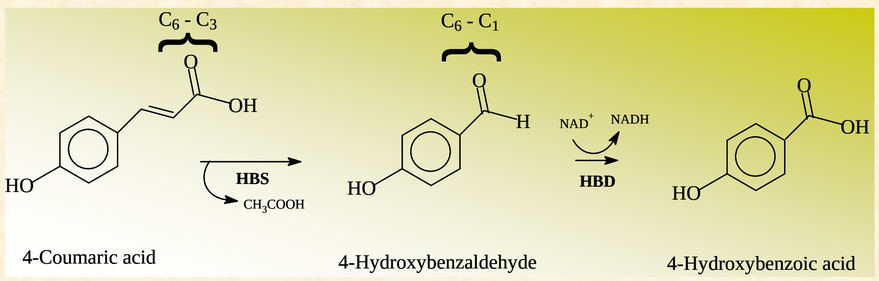


Fig.: Proposed enzymatic route of 4-hydroxybenzoic acid formation in hairy roots of *D. carota*

Fig.: Hairy roots of *D. carota*

Cloning of a core HBS cDNA:

Homology based cloning technique was used to amplify a putative core cDNA of HBS from hairy roots of *Daucus carota*.

Poly (A+) mRNA for cDNA synthesis was isolated from elicited hairy roots of *D. carota* producing substantial amount of 4-hydroxybenzoic acid.

Due to cysteine protease nature of *Vanilla* HBS [4], degenerate primers were designed using conserved domains for senescence specific plant cysteine proteases (CP).

These primer combinations led to the amplification of a 390 bp fragment that show 82% identity with vanilla HBS and 84% with senescence specific CP from *D. carota.*

Currently, work is in progress to make a full length clone of HBS cDNA using RACE-PCR

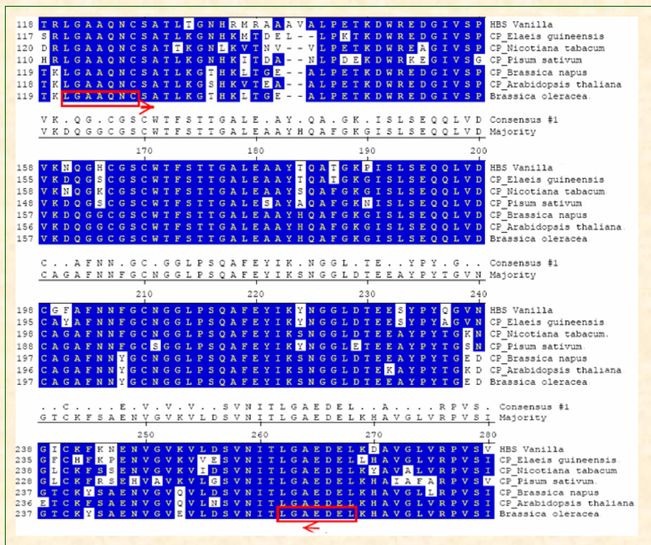


Fig.: Alignment of plant cysteine protease sequences

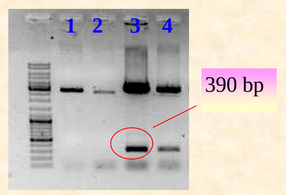


Fig.: 390 bp long HBS core fragment (after RE-digestion)
1, 2 - control; 3,4 - elicited

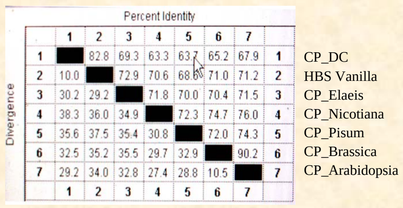


Fig.: Percent identities

The last enzymatic step in 4-hydroxybenzoic acid biosynthesis of *D. carota* was catalyzed by NAD^+ dependent HBD enzyme that catalyzes dehydrogenation of 4-hydroxybenzaldehyde (product of HBS reaction) into 4-hydroxybenzoic acid. HPLC and GC-MS based assays were performed for HBD.

HBD enzyme was extensively characterized using desalted cell-free extract from elicited hairy roots. Apparent Km values for 4-hydroxybenzaldehyde and NAD+ are 54.8 µM and 74.8 µM, respectively.

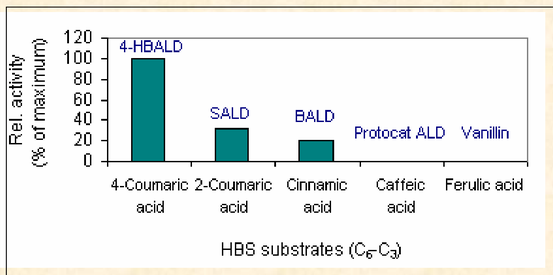


Fig.: Substrate specificity of HBS

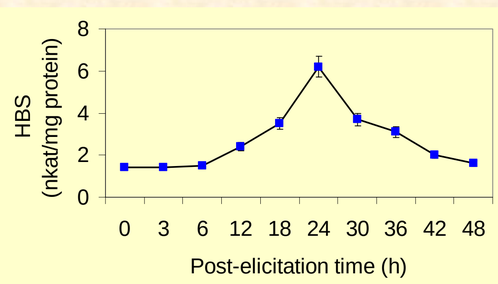


Fig.: Methyl-jasmonate induced time-course changes in HBS activity.

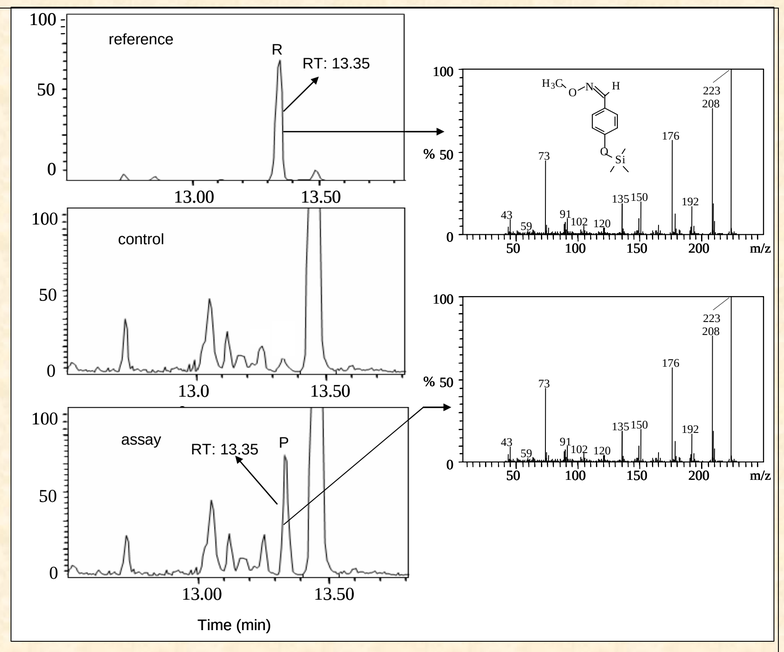


Fig.: GC-MS analysis of HBS assay. Peak corresponding to RT = 13.35 represents 4-hydroxybenzaldehyde (product of HBS)

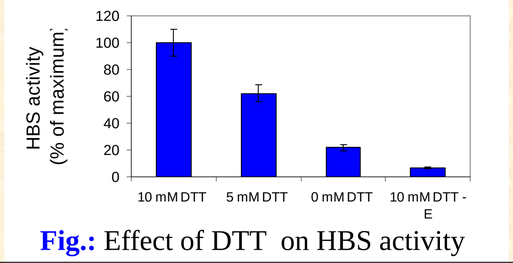


Fig.: Effect of DTT on HBS activity

Table.: Optimum conditions for HBS assay

pH	Inc. Temp	Inc. Time	Protein linearity
7 - 8.5	35 ^{0}C	30 min	50 µg/ assay

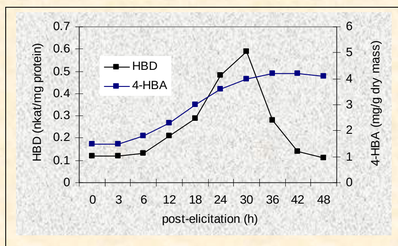
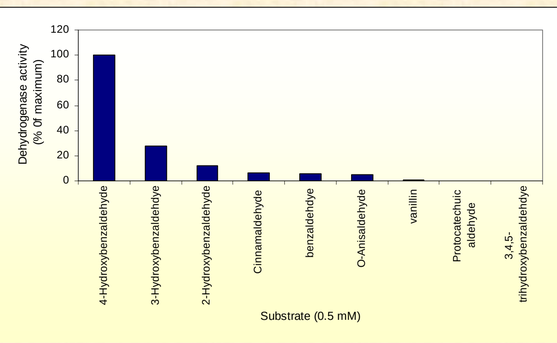


Fig.: Methyl-jasmonate induced Time-course changes in HBD activity.



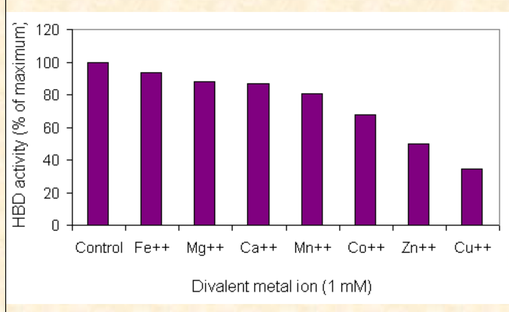


Fig.: Substrate specificity of HBD

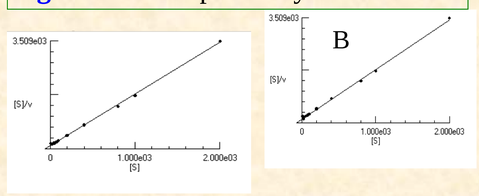


Fig.: Apparent values of *Km* from Hanes plot. (A): 4-HBALD, (B): NAD^+

[1] Wildermuth MC. Curr Opin Plant Biol 2006;9:288-96
[2] Sircar D, Mitra A. J Plant Physiol 2008;165:407-14.
[3] Sircar D, Mitra A. J Plant Physiol 2009; **166**: 1370-80
[4] Havkin-Frenkel, D., Podstolski, A., and Dixon, R.A . (2003)US patent No.: 20030070188

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