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BIOCHEMICAL AND GENETIC CHARACTERIZATION OF AN ACID PHOSPHATASE/5'-NUCLEOTIDASE FROM SOYBEAN ROOT NODULES

by

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A DISSERTATION

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5'-nucleotidase from Soybean Root Nodules

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BIOCHEMICAL AND GENETIC CHARACTERIZATION OF AN ACID PHOSPHATASE/5'-NUCLEOTIDASE FROM SOYBEAN ROOT NODULES

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University of Nebraska, 1998

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Soybeans and other tropical legumes assimilate fixed nitrogen into ureides. The first and last stages of ureide biosynthesis have been well studied; however, the intermediate reactions, whereby IMP is converted to xanthine, are still poorly understood. A soluble phosphatase (ACP) from soybean root nodules was purified which exhibits highest specificity for 5'-nucleotides and is postulated to dephosphorylate 5'-XMP and/or 5'-IMP in the intermediate reactions of ureide biosynthesis. A cDNA encoding ACP was isolated, and the mRNA was shown to be dramatically nodule-enhanced and developmentally regulated in a manner consistent with a role in ureide biosynthesis. The enzymatic properties of ACP were confirmed by heterologous expression in *Pichia pastoris*. The relationship of the nodule ACP to plant phosphatases, vegetative storage proteins, and the HAD superfamily of alpha-beta hydrolases is discussed.

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ABBREVIATIONS

ACP, acid phosphatase

AOX, alcohol oxidase

BMMY, buffered complex methanol medium

BMGY, buffered complex glycerol medium

CM, carboxymethyl

CV or col-vol, column volume

E-64, L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane,-[*N*-(L-3-transcarboxyirane-2-carbonyl)-L-leucyl]-agmatine

FMN, flavin mononucleotide

HTP, hydroxyapatite

MD, minimal dextrose

MM, minimal methanol

PNPP, para-nitrophenylphosphate

TFA, triflouroacetic acid

General Introduction

Root nodules are the result of a symbiotic relationship between a plant and a nitrogen fixing bacteria. All of the experiments in this project use nodules from the soybean (*Glycine max*)/*Bradyrhizobium japonicum* symbiosis.

Rhizobial attraction to soybean

The association of *Bradyrhizobium japonicum* and soybean is initiated by a chemoattraction mediated by plant-exuded flavonoids (van Rhijn and Vanderleyden, 1995). A variety of flavonoids and isoflavonoids, which are derived from phenylpropanoid metabolism, are found in soybean exudate. A characterization of soybean seedling, seed, and root exudates by Graham (1991) revealed an abundance of the isoflavones daidzein and genistein as well as several glycosyl derivatives of these isoflavones. The simplest of these derivatives are the 7-*O*-glucosyl conjugates, called daidzein and genistin. Other derivatives are malonylated conjugates of daidzein and genistein termed MGD and MGG, respectively. Various flavonoids have been tested for their ability to initiate *B. japonicum* response (reviewed by van Rhijn and Vanderleyden, 1995). The aglycosylated forms daidzein and genistein were found to be the most potent

inducers. However, it should be noted that the glycosyl derivatives have a higher solubility in water, and rhizobia secrete a variety of glycosidases which can convert the glycosyl derivatives into daidzein and genistein (van Rhijn and Vanderleyden, 1995).

Rhizobia nodulation genes

Bradyrhizobium japonicum responds to the plant-exuded isoflavones by the up-regulation of a host of genes involved in nodulation (reviewed by Denarie and Debelle, 1996). These genes specifically responsible for the initiation of nodulation are called *nod* genes. Apparently there are two concerted pathways for rhizobial response to the soybean isoflavones. One of these responses involves the *nodV* and *nodW* genes (Goffert et al., 1990; Sanjuan et al., 1994). *nodV* and *nodW* encode members of the two-component signal transduction family. *nodV* encodes a protein which presumably responds to an environmental stimulus and transduces that signal to *nodW*, which is likely a transcription factor responsible for the up-regulation of certain gene(s) involved in the initiation of nodulation. The second pathway for isoflavone response, which is much better understood, involves the *nodD* gene. *nodD* is a member of the LysR family of transcription factors and is activated

by the binding of isoflavones (van Rhijn and Vanderleyden, 1996). NodD then activates the transcription of a host of *B. japonicum* genes that possess a promoter element for NodD binding. This promoter element has been termed the *nod* box.

B. japonicum nod box genes

The majority of Rhizobial *nod* box genes encode enzymes which are responsible for the synthesis and secretion of lipochitooligosaccharide (LCO) signaling molecules (nod factors). The *nod* box genes can be divided into three classes based on the enzymatic properties of the proteins they encode. The first class of *nod* box genes is called *nodABC* or "common" *nod* genes because they are common to all rhizobia. The NodA, NodB, and NodC proteins are responsible for the synthesis of a chito oligosaccharide backbone which serves as the structural scaffold of the LCOs secreted by all rhizobia. NodA is an N-acetyltransferase, NodB is a de-N-acetylase, and Nod C is a UDP-GlcNac-transferase (Denarie and Debelle, 1996). The second class of *nod* box genes are the *nodI* and *nodJ* genes which encode an ATP-binding protein and a membrane protein, respectively (Denarie and Debelle, 1996). *nodI* and *nodJ* are also common to all rhizobia and are responsible for the secretion of LCOs. The third class of *nod* box genes encode enzymes which catalyze species- or

strain-specific modifications of LCOs. These modifications may be either N-substitutions or O-substitutions at several sites on the LCO. The B. japonicum *nodS*, *nodU*, and *nodZ* encode S-adenosyl methionine methyl transferase, 6-O-carbamolyltransferase, and fucosyl transferase, respectively (Denarie and Debelle, 1996). The concerted action of the rhizobia *nod* box gene products results in the secretion of a species- or strain-specific LCO.

Early nodulins

Several legume genes have been identified which are expressed early in nodulation in response to LCOs. The expression of the early nodulin, or *enod*, genes can be spatially separated into two classes. The first class are those specific to the epidermal cells (Scheres et al., 1990). Most of these genes are presumed to be involved in formation or addition of new cell wall material for the developing nodule. Two examples of epidermal *enod* genes are *enod5* and *enod12* (reviewed by Denarie and Debelle, 1996) which are postulated to encode cell wall proteins. These genes are turned on in response to very low concentrations of LCO (10^{-12}-10^{-13} M) and require the entire cognate LCO for activation; substitutions and deletions in the LCO are not tolerated. The other type of *enod* genes are those expressed

in the stele (root vascular tissue and surrounding pericycle). The only extensively studied member of this class is *enod40*. *enod40* mRNA accumulates in the pericycle within hours after treatment with LCO.

Ureide biosynthetic pathway

A host of other nodulins, or nodule-enhanced proteins, are expressed later in the developed nodule. Many of these nodule-enhanced genes encode enzymes of the ureide biosynthetic pathways. Soybeans and other tropical legumes export fixed nitrogen in the form of ureides, allantoin and allantoic acid. The first stage (IMP biosynthesis) and the last stage (xanthine oxidation to ureide) of the ureide biosynthetic pathway have received considerable attention. All of the ten enzymes of IMP biosynthesis have been found to be localized to both mitochondria and plastids of the rhizobia-infected cells (Atkins et al., 1997). Genetic complementation of *E.coli* purine auxotrophs has facilitated the isolation of cDNAs encoding the first three enzymes in the purine biosynthetic pathway: phosphoribosylpyrophosphate amidotransferase (PRAT; Kim et al., 1995); glycinamide ribonucleotide (GAR) synthetase (Schnor et al., 1996); and GAR transformylase (Schnor et al., 1996). Expression of all three genes is markedly enhanced in root nodules in

comparison to other tissues and developmentally regulated in the nodule. The enzymes of xanthine oxidation have been examined by immunological localization. XDH was found predominantly in the uninfected cell cytoplasm (Datta et al., 1991), while uricase was found in the peroxisomes of uninfected cells (Van den Bosch and Newcomb, 1986). Takane et al. (1997) demonstrated that there are two uricase II genes in soybean. One of the genes, *UR2*, is nodule enhanced, while the other, *UR9*, is expressed at a low level in most tissues. The intermediate pathway, whereby IMP is converted to xanthine, presumably in the infected cell cytoplasm, is still poorly understood. The intermediate reactions have been difficult to study in part because nodule extracts possess at least two concentration-dependent pathways of IMP catabolism, one through the oxidation of IMP to XMP and another through the dephosphorylation of IMP (Shelp and Atkins, 1983). IMP dehydrogenase and a nucleosidase active against both inosine and xanthosine have been isolated from cowpea nodules, but no protein sequence data, cDNAs, or antibodies are available. The nucleotidase in the pathway has been particularly difficult to study since IMP and XMP are likely substrates for many phosphatases in a nodule extract.

The research reported in this dissertation was

initiated to biochemically and genetically characterize a new nodule-enhanced phosphatase from soybean root nodules that may participate in the intermediate reactions of the ureide biosynthesis.

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Chapter 1

Soybean Root Nodule Acid Phosphatase

Abstract

Acid phosphatases are ubiquitous enzymes that exhibit activity against a variety of substrates in vitro, although little is known about their intracellular function. In this study we report the isolation, characterization, and partial sequence of the major soybean root nodule acid phosphatase. The phosphatase was purified predominantly as a heterodimer with subunits of 28 and 31 kDa; homodimers of both subunits were also observed and exhibited phosphatase activity. In addition to the general phosphatase substrate p-nitrophenyl phosphate, the heterodimeric form of the enzyme readily hydrolyzed 5′-nucleotides, flavin mononucleotide, and O-phospho-L-tyrosine. Low or negligible activity was observed with ATP or polyphosphate. Purified nodule acid phosphatase was stimulated by Mg^{2+}, inhibited by Ca^{2+} and EDTA and competitively inhibited by cGMP and cAMP with apparent Ki values of 7 and 12 μM, respectively. Partial N-terminal and internal sequence of the nodule acid phosphatase revealed homology to the soybean vegetative storage proteins. There