PURIFICATION AND CHARACTERIZATION OF UDP-GLUCOSE:SINAPIC ACID GLUCOSYLTRANSFERASE FROM BRASSICA NAPUS SEEDLINGS

Shawn X. Wang[1], Thomas Vogt[2] and Brian Ellis[1]

[1] Department of Plant Science, University of British Columbia, Vancouver, BC, V6T 1Z4 CANADA
[2] Institute of Pflanzenbiochemie, Halle, Germany

ABSTRACT

UDP-glucose:sinapic acid glucosyltransferase (SGT) is one of the key enzymes involved in biosynthesis of sinapine in *Brassica napus* and other cruciferous species. SGT has been purified 134-fold from 65-h-old seedlings of *B. napus* cv. Westar and found to have a native Mr of 42,500 and a pI of 5. At its optimum pH of 6.0, SGT showed a Km for UDPG of 0.24 mM and for sinapic acid of 0.16 mM. The enzymatic mechanism fits the random bi-bi mode. SGT activity could be detected in all growth stages of *B. napus* plants, but was most active in juvenile tissues and developing seeds.

INTRODUCTION

Sinapine, a phenolic ester of sinapic acid and choline, makes up about 2% of the air-dried oil-free canola/rapeseed meal. Elimination of sinapine from the seeds would improve the flavor, palatability and nutritional properties of canola meal and thereby enlarge markets for canola. Sinapine content in seed may vary from species to species, and from cultivar to cultivar within *Brassica* (Mueller et al. 1978; Wang, 1992) but variation is quite limited (17.3 - 21.6 mg/g meal). An alternative route to low sinapine germplasm is through genetic engineering. The biosynthesis of sinapine is a secondary metabolic process during seed development. Enzymatic synthesis studies in *Raphanus* indicated that sinapic acid could not be directly used to synthesize sinapine. Sinapic acid had to first be activated by the formation of the glucose ester of sinapic acid (1-O-sinapoyl-β-D-glucose, SinG) from UDPG and sinapic acid. This reaction is catalyzed by UDPG:sinapoyl glucosyltransferase (Strack, 1980; Nurmann and Strack, 1981). The SinG provides the acyl donor for the synthesis of sinapine, which is catalyzed by SinG:choline sinapoyltransferase (SCT). Our ultimate objective is to use antisense technology to create a new genotype of *B. napus* with a greatly reduced content of sinapine in the seeds. As the first step towards gaining access to the SGT gene, we are purifying SGT and investigating SGT expression during plant development.

MATERIALS AND METHODS

B. napus cv. Westar was obtained from Agriculture Canada, Saskatoon Research Station. Sinapic acid was purchased from Aldrich Chemicals. All of other chemicals was from Sigma. The 65-h-old seedlings were frozen with liquid nitrogen and then ground with a coffee mill. The fine powder was extracted with chilled 50 mM Tris buffer, pH 7, including 5% glycerol, 4% PPVP and 10 mM 2-mercaptoethanol (2-ME). Desalted protein was used for a series of chromatography (see Table 1). All buffers contain 5% glycerol and 10 mM 2-ME or 2 mM DTT. Westar plants grown in the greenhouse were separated into different tissue types and analyzed at all growth stages. Tissue samples were extracted with Tris buffer. After removing tissue residue by centrifugation, the supernatant was used for SGT activity assay. The SGT activity was assayed by HPLC on a 0.46 x 250 cm Hypersil RP MOS 5 μ HPLC column (Alltech) and the eluent absorbance was measured at 350 nm.

RESULTS AND DISCUSSION

Purification

SGT from 65-h-old *B. napus* seedlings was purified 134-fold with an overall yield of 0.5% according to the 9-step purification procedure listed in Table 1. The purified SGT was not homogeneous at this point. The protein profile on a non-SDS 10% PAGE gel showed one major and two minor protein bends after silver staining.

Table 1. Purification of SGT from 65-h-old seedlings of *B. napus* cv. Westar

Purification step	Total Activity (pKat)	Specific Activity (pKat/mg protein)	Total protein (mg)	Enriched fold
Crude extract	42743	19.4	2200	1.0
Protamine sulfate	41353	28.2	1465	1.5
60% Ammonium sulfate	15380	59.8	257	3.1
Prep-Mono Q (anion exchange)	11571	100.6	115	5.2
Hydroxyapatite	8762	190.5	46	9.8
EAH-SinA affinity	6482	308.7	21	15.9
Mono Q (anion exchange)	5714	317.4	18	16.3
Mono P (Chromatofocusing)	3651	688.9	5.3	35.5
Superose 12 (gel filtration)	1373	1961.4	0.7	101.0
Reverse immunoaffinity	235	2611.1	0.09	134.4

Physical and chemical properties

The general characteristics of partially purified *Brassica* SGT are summarized in Table 2. This enzyme can catalyze the reaction for both directions: SinA + UDPG ⇔ SinG + UDP. Most of *Brassica* SGT characteristics are similar to the enzyme isolated from *R. sativus*. However, the kinetic properties of SGT from *B. napus* suggests that its catalytic mechanism fits the random bi-bi model, whereas the enzyme from *R. sativus* appeared to use an ordered bi-bi mechanism (Mock and Strack, 1993).

Table 2. Characteristics of SGT from 65-h-old seedlings of *B. napus* cv. Westar

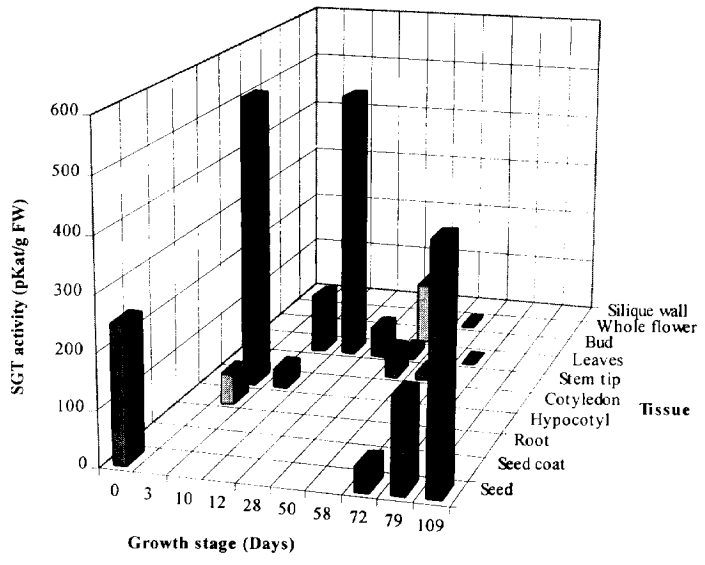
Item	Properties
MW	42.5 KDa (Native)
Isoform	Not detected
pI	pH 5
Optimum pH	pH 6 (100 mM MES), active at pH 5 - 8 and stable at pH 6 - 7.
Optimum temperature	32 °C, active at 4 - 42 °C and stable at < -20 °C.
Co-factor	Not detected
Divalent ion	Not required, sensitive to Zn^{++}, Cu^{++}, Hg^{++}, Fe^{++}, Co^{++}, inhibition by Zn^{++}, Co^{++} and Fe^{++} was reversible with EDTA, but not Cu^{++} and Hg^{++}
Reducing reagent	DTT, 2-ME required for stability, but not ascorbic acid.
Inhibitor	PHMB showed inhibition, but not NEM, IAA
Analogue inhibition	UDP, TDP, UDP-mannose showed strong inhibition.
Relative substrate specificity	Sinapic acid 100, ferulic acid 77, 5-OH-ferulic acid 39, cinnamic acid 24, p-cumaric acid 21, caffeic acid 14, syringic acid 10 UDP-glucose 100, TDP-glucose 96
Reaction mechanism	Random bi-bi, $K_{[UDPG]}$ 0.24 mM, $K_{[sinapic\ acid]}$ 0.16 mM, V_{max} 10.6 pKat
Reversibility	UDP, TDP can be used to form UDPG and TDPG.
Subcellular location	Cytosol

Developmental expression of SGT

The developmental expression of SGT of *B. napus* cv. Westar is summarized in Figure 1. SGT specific activity in whole seedling tissue reached its peak between the 2nd and 3rd day, then decreased > 80%. This indicates that the SGT gene is highly expressed in the early stages of seed germination. The physiological function of SGT at this stage is very clear. Highly activated or newly synthesized SGT can rapidly convert sinapic acid released from sinapine to sinapoylglucose. When true leaves developed, the SGT level in the cotyledons declined dramatically. Stem tissue did not show SGT activity except at the apex. In mature plants, most of the SGT activity was found in the leaves; younger leaves showed higher SGT activity than older leaves but no activity in the petiole tissue. When *B. napus* plants went into reproductive stage, the majority of SGT activity was found in the floral bud and stem apex. During the seed

development stage, seed tissue was the only part of the plant which showed detectable SGT activity. SGT specific activity (pKat per mg of protein) reached its peak when the seed was green (after 26 days of flowering). Total SGT activity (pKat per g of fresh seed) continued to increase, but the specific activity gradually declined after the green seed stage, probably due to seed dehydration and accumulation of storage proteins.

Figure 1. SGT activity in different tissues of *B. napus* cv. Westar at different growth stages



ACKNOWLEDGMENTS

The authors thank the Natural Science and Engineering Research Council of Canada for the financial support.

REFERENCES

Mock, H.-P and Strack, D. 1993. Energetics of the uridine 5'-diphosphoglucose:hydroxy-cinnamic acid acyl-glucosyltransferase reaction. *Phytochemistry*, **32**, 575-579.

Mueller, M.M., Ryl., E.B., Fenton, T. and Clandinin, D.R. 1978. Cultivar and growing location differences in the sinapine content of rapeseed. *Canadian Journal of Animal Science*, **58**, 579-583.

Nurmann, G and Strack, D. 1981. Formation of 1-sinapoylglucose by UDP-glucose:sinapic acid glucosyltransferase from cotyledons of *Raphanus sativus*. *Z. Pflanzenphysiol.*, **102**, 11-17.

Strack, D. 1980. Enzymatic synthesis of 1-sinapoylglucose from free sinapic acid and UDP-glucose by a cell free system from *Raphanus sativus* seedlings. *Z. Naturforsch.*, **35**, 204-208.

Wang, X. 1992. Studies of methodology of sinapine determination and sinapine variation in *Brassica* and *Sinapis* species. M. Sc. thesis of Department of Plant Science and Plant Ecology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada pp. 114-123.