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Stabilization of dhurrin biosynthetic enzymes from *Sorghum bicolor* using a natural deep eutectic solvent

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**ABSTRACT**

In recent years, ionic liquids and deep eutectic solvents (DESs) have gained increasing attention due to their ability to extract and solubilize metabolites and biopolymers in quantities far beyond their solubility in oil and water. The hypothesis that naturally occurring metabolites are able to form a natural deep eutectic solvent (NADES), thereby constituting a third intracellular phase in addition to the aqueous and lipid phases, has prompted researchers to study the role of NADES in living systems. As an excellent solvent for specialized metabolites, formation of NADES in response to dehydration of plant cells could provide an appropriate environment for the functional storage of enzymes during drought. Using the enzymes catalyzing the biosynthesis of the defense compound dhurrin as an experimental model system, we demonstrate that enzymes involved in this pathway exhibit increased stability in NADES compared with aqueous buffer solutions, and that enzyme activity is restored upon rehydration. Inspired by nature, application of NADES provides a biotechnological approach for long-term storage of entire biosynthetic pathways including membrane-anchored enzymes.

1. Introduction

Today, we rapidly need to move into The Planthropocene Era where petrochemicals are replaced by sustainable green bio-production (Møller, 2014, 2017). Green photosynthetic organisms are instrumental in this transition. Using solar energy as the energy source and carbon dioxide from the atmosphere as the sole carbon source, photosynthetic organisms provide food, biomaterials and bioenergy. At a minor scale, they also serve as a rich source of bio-active natural products used by humanity as nutraceuticals, condiments, flavors, colorants as well as medicinal compounds (Jensen and Scharff, 2019). These precious natural products are typically found in small amounts and are present in native plants that are not easily grown at a commercial scale. This renders harvest and extraction unsustainable.

Some unique plant species have the ability to accumulate extraordinarily high levels of natural products stored in soluble form in their tissues. In the black flower petals of the gentiana “flower of death” (*Listanthus nigrescens* Schltdl. & Cham.), the anthocyanin content constitutes 24% of the dry mass (Markham et al., 2004). In the buds and flowers of sophora (*Sophora japonica* L, Pagoda tree), the content of the anthocyanin rutin constitutes up to 30% of the dry mass (Chen et al., 2018; Koja et al., 2018). This is equivalent to a 12,000 fold higher solubility in the plant tissue in comparison to aqueous media (Dai et al., 2016; Horosanskaia et al., 2017). Anthocyanins may self-organize and accumulate in anthocyanoplants or in anthocyanic vacuolar inclusions, which may or may not be membrane encapsulated (Chanoca et al.,...
Raisins maintain a liquid phase despite an almost complete removal of a highly viscous syrup, as found in raisins (Grimplet et al., 2009). Compared to their individual components, resulting in the formation of a mixture of glucose:tartrate in a 1:1 M ratio (approx. 8% water and then lyophilizing until the mass remains constant. This applies for a mixture of glucose and tartrate constituting 30% of the dry mass (Halkier and Møller, 1989; Kojima et al., 1979). Dhurrin had previously been reported to be stored in the main vacuole (Saunders and Conn, 1978), but this could not be verified by Raman spectrometry, which pointed to storage in the cytosol or apoplast (Heraud et al., 2018) i.e. in a more confined space that would demand storage at molar concentrations. These examples demonstrate that a wide range of plant species are able to orchestrate the establishment of high-density distinct storage sites where natural products with sparse water solubility are kept in soluble form at surprisingly and seemingly impossible high molar concentrations in confined cellular domains. In nature, specific plant species thus possess an inherent ability to biosynthesize, accumulate and store large quantities of bio-active natural products. The origin and physical characteristics of these conspicuous storage structures remain controversial. If the biological mechanisms orchestrating these abilities were known, they might be engineered into commercially grown plants using synthetic biology approaches.

Verpoorte and co-workers have proposed that Natural Deep Eutectic Solvents (NADESs) could be involved in establishing high-density biocondensates (Choi et al., 2011; Knudsen et al., 2018). The NADES concept was coined based on nuclear magnetic resonance (NMR) spectroscopy studies demonstrating the presence of sugars, protein amino acids, choline and organic acids in plant cells (Fig. 1A). A NADES forms a highly viscous liquid - a liquid crystal - upon mixing specific constituents in the right stoichiometric proportions, dissolving in water, and then lyophilizing until the mass remains constant. This applies for a mixture of glucose:tartrate in a 1:1 M ratio (approx. 8% water remaining) (Fig. 1B), which has a significantly lowered melting point compared to their individual components, resulting in the formation of a highly viscous syrup, as found in raisins (Grimplet et al., 2009). Raisins maintain a liquid phase despite an almost complete removal of water. NADESs composed of specific subsets of these crystalline constituents have been demonstrated to be excellent solvents for sparsely soluble natural products such as vanillin glucoside and rutin and related anthocyanins (Dai et al., 2013a, 2013b, 2015, 2016; González et al., 2018; Peng et al., 2018; Vetrova et al., 2017; Zhao et al., 2015). Accordingly, NADESs are being used as green solvents for the industrial-scale extraction of bio-active natural products from plants. However, no in planta functions of NADESs have been demonstrated, nor is the mechanism of their possible formation understood.

Sorghum (Sorghum bicolor (L.) Moench) (Poaceae) is a drought-tolerant cereal that maintains growth even under severe drought stress (Pavli et al., 2013). Metabolite profiling of sorghum plants exposed to different levels of drought revealed a significant accumulation of organic acids, sugars and amino acids. Simultaneous accumulation of such metabolites and depletion of water provides ideal conditions for the formation of intracellular NADES phases for safe-storage of both enzymes and natural products such as dhurrin. As stated above, sorghum seedlings accumulate high amounts of dhurrin, and increased amounts of dhurrin are produced in the mature plant upon exposure to drought stress (O’Donnell et al., 2013). Dhurrin is an insect defense compound that upon tissue disruption e.g. as caused by feeding insect larvae, is hydrolyzed by a β-glucosidase to release toxic hydrogen cyanide gas (Gleadow and Møller, 2014; Morant et al., 2008; Poulton and Li, 1994; Sanchez-Perez et al., 2009; Swain et al., 1992). Dhurrin also functions as a storage of nitrogen that can be remobilized by recycling pathways, thus avoiding hydrogen cyanide formation and providing an important source of reduced nitrogen at specific developmental stages (Bjarnholt et al., 2018; Picmanova et al., 2015). Dhurrin is synthesized from the amino acid L-tyrosine (Fig. 1C). The enzymatic conversion is catalyzed by a dynamic multi-component enzyme complex, the dhurrin metabolon (Bassard et al., 2017; Jørgensen et al., 2005; Laursen et al., 2016). The metabolon is composed of a cytochrome P450 oxidoreductase (POR) (Bavish et al., 2018; Laursen et al., 2014), two cytochromes P450 (CYP79A1 and CYP71E1) (Bak et al., 1998; Blomstedt et al., 2011; Kahn et al., 1999; Koch et al., 1995; Sibbesen et al., 1995) and a UDP-glucosyltransferase (UGT85B1) (Blomstedt et al., 2015; Jones et al., 1999). The membrane-bound CYP79A1 and CYP71E1 enzymes constitute less than 1% of the total membrane protein in sorghum seedlings, yet catalyze the formation of dhurrin up to 30% of the dry mass (Halkier and Møller, 1989; Sibbesen et al., 2015; Gallage et al., 2014).
The micro-environment surrounding the dhurrin metabolon including the lipid membrane is important for high flux through the metabolon, increasing the conversion of \( \text{\textit{L}} \)-tyrosine to the product dhurrin (Laursen et al., 2016). Likewise, the cytosolic micro-environment has been proposed to serve as a stabilizing solvent for dhurrin and prevent auto-hydrolysis at cytosolic pH (Knudsen et al., 2018; Møller et al., 2016). With the hypothesis that dhurrin is being stored in cytosolic or apoplastic high-density bio-condensates formed in a NADES-based environment, we aimed to examine whether the enzymes of the dhurrin metabolon would be active in such a hypothetical cellular environment. Additionally, we investigated the stability of the dhurrin metabolon in NADES, thereby mimicking conditions resulting from abiotic stresses such as dessication and heat. This study adds to the increasing evidence that a NADES-based cellular environment might represent a third intracellular phase that complements the aqueous and lipid phases in plant cells as suggested by Verpoorte and coworkers (2011).

2. Results

2.1. Enzyme activity in the presence of NADES

As stated in the introduction, NADESs are composed of common metabolites present in living organisms including plants. These include sugars, protein amino acids, choline and organic acids (Choi et al., 2011). In general, NADES consists of a hydrogen-bonding salt and a neutral species present in an eutectic molar ratio with a melting point that is much lower than those of each constituent (Dai et al., 2013b; Hammond et al., 2017). The most common eutectic mixtures are composed of an organic acid and a sugar, an organic acid and choline chloride, or choline chloride and sugar.

To select an appropriate NADES for the experimental work, initial biosynthetic experiments were performed using microsomes prepared from etiolated sorghum seedlings (Fig. 2A). Previous studies have demonstrated that the microsomes catalyze the conversion of \( \text{\textit{L}} \)-tyrosine to \( \text{\textit{p}} \)-hydroxymandelonitrile i.e. all the membrane-bound steps of the dhurrin pathway catalyzed by CYP79A1, CYP71E1 and POR2B. These analyses utilized classical tricine or phosphate buffers in the presence of NADPH and oxygen (Bak et al., 2000; Jones et al., 1999; Kahn et al., 1999; Laursen et al., 2011). Because the enzymes in the dhurrin pathway are organized within a metabolon, no accumulation of intermediates such as \( \text{\textit{p}} \)-hydroxyphenylacetaldoxime and \( \text{\textit{p}} \)-hydroxyphenylacetoneitrile is observed. The \( \text{\textit{p}} \)-hydroxymandelonitrile intermediate is labile and dissociates into \( \text{\textit{p}} \)-hydroxybenzaldehyde and hydrogen cyanide. Product formation from \( ^{13}\text{C-L} \)-tyrosine was monitored by autoradiography following separation using thin layer chromatography (TLC) (Kahn et al., 1999). In the present study, different NADES constituents were included in the assays to assess their possible effect on the biosynthetic enzymes. Maximal activity of the membrane associated metabolon, namely POR2B, CYP79A1 and CYP71E1, catalyzing the conversion of \( \text{\textit{L}} \)-tyrosine to \( \text{\textit{p}} \)-hydroxymandelonitrile, was observed at a 5% concentration of NADESs composed of glucose:tartrate and glucose:malate as monitored by the conversion of tyrosine (Fig. 2B). The NADES concentration of 5% is within the macromolecule concentration range of 5%–40% assumed to be present in the cytosol of healthy hydrated cells (Ellis and Minton, 2003). Enzymatic activity was strongly decreased at higher concentrations of these NADESs, such as 25% NADESs corresponding to 1 M of glucose:tartrate and 1.2 M glucose:malate. The reduced activity might be attributed to the high viscosity of the medium, which would restrict domain motion related to catalytic function (Laursen et al., 2011) and diffusion of substrates. The presence of choline chloride was found to have a stronger inhibitory effect on microsomal activity with total inhibition at and above 20%, corresponding to 0.5 M glucose and 1 M choline in the 1:2 NADES (Fig. 2B). The focus was therefore shifted to NADESs composed of an organic acid and a sugar. In these NADES systems, salts of simple organic acids such as tartaric acid, malic acid, maleic acid and malonic acid are common. We chose to proceed with a NADES composed of glucose as the sugar and tartaric acid as the organic acid since these two constituents displayed minimal effect on the enzyme activity when present at 12%, corresponding to 0.45 M of glucose:tartrate and 0.58 M glucose:malate, as required for studying the recovery of enzymatic activity upon storage in high NADES concentrations followed by dilution.

2.2. NADES-based stabilization of the dhurrin biosynthetic enzymes

In order to investigate the ability of the glucose:tartrate NADES to preserve the dhurrin synthesizing enzymes during heat and desiccation stress, we expressed the genes encoding CYP79A1 and CYP71E1 in Saccharomyces cerevisiae and UGT85B1 and POR2B in Escherichia coli, isolated the proteins formed and reconstituted CYP79A1, CYP71E1 and POR2B in proteoliposomes composed of native lipids extracted from etiolated sorghum seedlings (Laursen et al., 2016) (Fig. 3A). The stability of the enzymes in the proteoliposomes was tested by incubation in high NADES concentrations (72%) followed by dilution to 12%. Upon dilution of the NADES solution, 82% of the initial tyrosine conversion rate was recovered (Fig. 3B). Glycerol at high concentrations was included as a control for crowding effects (Ellis and Minton, 2003) and enzymes in glycerol displayed 61% recovery of activity upon dilution from 72% (7.8 M) to 12% (1.3 M). The UGT85B1 recovered approximately 70% of the initial activity upon dilution of the NADES as monitored following administration of \( \text{\textit{p}} \)-hydroxymandelonitrile and radiolabeled UDP-glucose (Fig. 3B). This demonstrates that NADES may provide an inert environment protecting against irreversible
Fig. 3. NADES-based stabilization of the dhurrin biosynthetic enzymes. A) Illustration of proteoliposomes comprising the POR2B, CYP79A1, CYP71E1 and UGT85B1 reconstituted in liposomes composed of phospholipids extracted from etiolated sorghum seedlings (Metabolon). B) Recovery of activity upon storage of enzymes in NADES and glycerol compared to buffer upon dilution displayed as relative conversion of tyrosine for the Metabolon samples and conversion of cyanohydrin to dhurrin for the UGT85B1 samples. Values are mean of three technical replicates ± SD. C) Stability of dhurrin biosynthetic enzymes stored at room temperature in aqueous buffer, NADES and glycerol. Samples were diluted in buffer prior to activity assay. Values are mean of three technical replicates ± SD and fitted to a double exponential decay. D) Bar plot showing relative activity of the enzymes following incubation at various temperatures for 30 min in aqueous buffer, NADES and glycerol. Samples were diluted in buffer prior to activity assay. All values are mean of three independent technical replicates ± SD.
denaturation or inhibition of proteins.

To further test the ability of NADES to stabilize enzyme activity during long-term storage, the dhurrin biosynthetic enzymes reconstituted in proteoliposomes were kept at room temperature in the concentrated glucose:tartrate NADES, glycerol or in aqueous buffer prior to dilution and activity assays. The activity of all proteins followed a double exponential decay indicating two phases of protein denaturation (Fig. 3C). CYP79A1 and CYP71E1 maintained activity for more than 30 d when stored in glucose:tartrate NADES compared to 5 d in aqueous buffer and 15 d in glycerol. Assays were performed by administration of tyrosine as substrate. Hence, the dramatic decrease of CYP71E1 activity is the cumulative effect of stability and the coupling between CYP79A1 and CYP71E1. UGT85B1 was more stable in glycerol compared to both the NADES and aqueous buffer, maintaining activity for more than 200 d (Fig. 3C). The temperature stability of the dhurrin pathway enzymes was monitored in the glucose:tartrate NADES, in glycerol and in aqueous buffer to assess whether the NADES would also protect against heat stress. All enzymes showed increased stability in the NADES at higher temperatures, resulting in at least 50% activity preservation following exposure to 60 °C for 30 min (Fig. 3D).

3. Discussion

3.1. Formation of NADES in plants and possible applications

In the present study we demonstrated that a glucose:tartrate NADES serves as an excellent in vitro stabilizing solvent for the dhurrin metabolon and possibly dhurrin. In the plant tissue, storage of dhurrin in high-density bio-condensates composed of a glucose:tartrate or similar based NADESs is thus a possibility. NADESs can be formed from general metabolites such as sugars, amino acids and organic acids, all of which are present in considerable amounts in the cells of all living organisms (Choi et al., 2011). Sugars serve the purpose of energy storage, but the reason that many of the other simple metabolites are present in high amounts has remained unresolved. Small organic molecules such as proline, trehalose and sucrose are known to contribute to freezing tolerance in wheat (Kovacs et al., 2011) and a number of sugars and amino acids have been implicated in drought tolerance (Hockstra et al., 2001). Based on the pioneering research from the groups of Robert Verpoorte and Young Hae Choi at Leiden University, subsets of many additional organic metabolites have been suggested to function as NADES providing, a third liquid phase for storage of high local concentrations of natural products in plant cells (Choi et al., 2011; Dolgin, 2018). Cellular constituents uncovered by NMR-based metabolomic analyses were found to be able to possibly form more than 30 combinations of liquid NADESs (Choi et al., 2011). Bio-production of high value natural products in engineered cells of cyanobacteria, algae or plants is currently challenging, being hampered by bottleneck issues such as low production levels of the desired bio-active natural product, autotoxicity and storage capacity issues. Storage within NADES-based compartments might overcome some of these bottlenecks and facilitate the path to the biological production of high value molecules based on solar energy and carbon dioxide from the atmosphere as the sole carbon source.

3.2. In vitro stabilization of the dhurrin biosynthetic enzymes in NADES

Using the enzymes in the biosynthetic pathway for the cyanogenic glucoside dhurrin as model enzymes in natural product metabolism (Fig. 1C), we investigated the ability of equimolar glucose:tartrate NADESs to stabilize the two cytochromes P450 CYP79A1 and CYP71E1, the UDP-glucosyltransferase UGT85B1 and the cytochrome P450 oxidoreductase POR2B (Fig. 3). Optimum enzyme activity was found at 5% NADESs (Fig. 2B), which suggests that the cellular interior may represent a dilute NADES environment (Ellis and Minton, 2003). At high concentration of the glucose:tartrate NADESs, conversion of tyrosine into dhurrin was negligible. However, the activity of the pathway enzymes was recovered by dilution of the NADES (Figs. 2 and 3). This demonstrates that the inactivation of the biosynthetic enzymes at the high concentrations of the glucose:tartrate NADES was reversible, despite almost complete removal of water from the system. Substitution of the water shell by sugars upon desiccation preserved the biosynthetic machinery. Combined with the increased heat stability of each of these enzymes in the glucose:tartrate NADES when compared with aqueous buffer (Fig. 3C and D), this provides further evidence that NADES are capable of fulfilling the role of mediating heat, dehydration and drought tolerance. Interestingly, the soluble enzyme UGT85B1 catalyzing the final step in the dhurrin biosynthetic pathway was only marginally influenced by the high concentrations of glucose in the glucose:tartrate NADES (Fig. 3B). This demonstrates that glucose when present in NADES does not compete with the UDP-glucose binding site of UGT85B1. Regardless of enzyme function, the presence of NADES as a third phase during desiccation is capable to prevent precipitation of enzymes, metabolites and polymers and can preserve enzyme function until the water balance is restored.

3.3. NADES phases assembled by intracellular liquid-liquid phase separation

NADESs form a “liquid crystal” through formation of inter- and intramolecular hydrogen bonds. This results in a high melting point depression causing the solids to liquidy and, in many cases, to remain fluid at room temperature. The unparalleled solubilization capacity of NADES would suggest that certain combinations of NADES constituents also possess the ability to establish hydrogen bonds to specific bio-active natural products. The water molecules present in NADES are highly coordinated by solvophobic sequestration, forming nanos-structured domains and worm-like structures orchestrated by the eutectic pair. The ionic clusters are part of a complex and structurally disordered hydrogen-bonding network. In this way, the NADESs may function as super-molecules mimicking the polymers known to stabilize liquid-liquid phase separation in animal cells (Bianani et al., 2017; Brangwynne et al., 2009; Gall, 2003; Pederson, 2011). Analyses using a range of different scattering techniques demonstrated that NADESs formation is not based on classical phase separation (Hammond et al., 2017). Hence the properties of NADESs are retained by dilution with up to 40–50 mass % H2O. Upon further addition of water, the unique properties of the NADES are gradually abolished.

3.4. Biological relevance of intracellular NADES phases

Our data support that NADESs may have an in vivo function in plants and other organisms, by establishing structurally confined high-density NADES-based bio-condensates, in which the biosynthetic enzymes and bio-active natural products are stored in a soluble form. The inherent properties of NADESs could enhance plant robustness because a NADES has almost no vapor pressure and thus retains water during desiccation caused by drought stress and heat waves. That is, in instances where dehydration of cells would result in protein precipitation or denaturation, the formation of NADESs and presence of sequestered water molecules may provide an environment in which proteins can maintain solubility and intramolecular structures until rehydration occurs.

Independent of whether a NADES phase is membrane encapsulated or not, NADES-based liquid phase separation would prevent osmotic issues related to accumulation of molar concentrations of natural products and thus prevent disruption of cell homeostasis (Aguilera-Gomez and Rabouille, 2017; Courchaine et al., 2016; Kuchler et al., 2016). In addition to providing storage capacity for bio-active compounds, NADES-based assemblies may also serve to mediate and fine-tune numerous biological processes. A higher order of metabolic and catalytic control may be achieved by limiting or improving enzyme
access and activity toward their substrates or by embedding entire biosynthetic pathways in NADES-based assemblies. The high solubilizing capacity of the NADES would imply that the biosynthesis of some natural products sparsely soluble in water could occur in NADESs, in which both substrate and enzymes are dissolved. Typically, \( K_m \) values are determined in dilute aqueous buffers. Such values may not be very relevant for enzymes exhibiting their catalytic functions in a NADES.

Dehydration of bio-active natural products and/or enzymes stored in NADESs might prevent some enzymatic conversions. NADESs may thus have multiple roles in the regulation, production and storage of natural products in addition to their ability to function as exquisite solvents. Formation of phase-separated NADES droplets in plant cells may thus contribute to the biosynthesis and compartmentalized storage of natural products.

3.5. Applications of NADES inspired by nature

In parallel to the implications of our studies, specific NADESs have been demonstrated to function as excellent storage media for human interferon-alpha 2a, an important therapeutic protein. Enzyme activity, long-term storage as well as resistance to denaturation at high temperatures were improved (Lee et al., 2018). Likewise, lipases were shown to possess dramatically enhanced activities in a NADES (Elgharbawy et al., 2018). NADESs based on trehalose and glycerol are being used as cryoprotective agents based on their strong effects on the water crystallization/freezing and melting process, resulting in the formation of a reduced number of ice crystals and hence reduced ice crystal damage in cells; a crucial parameter for their survival following freezing and thawing (Castro et al., 2018). The three-subunit membrane-spanning photosynthetic reaction center from Rhodobacter sphaeroides was shown to be stabilized in choline-based NADESs (Milano et al., 2017). The photosynthetic reaction center complex retained its activity upon illumination to carry out charge separation and reduce a quinone acceptor using cytochrome c as the electron donor.

In conclusion, our data on the ability of the glucose:tartrate NADES to stabilize long-term activity and heat-tolerance of the membrane-bound and soluble enzymes in \( \delta \)-dhurrin biosynthesis adds to the proposed role and importance of NADESs in biological systems.

4. Experimental

4.1. Chemicals

All chemicals were of analytical grade and purchased from Sigma Aldrich (Denmark) unless stated otherwise. \( \delta \)-dhurrin was chemically synthesized as previously described (Møller et al., 2016).

4.2. Preparation of NADES

The three different NADESs, composed of 1:1 D-glucose and dipotassium tartrate (glu:tar), 1:1 D-glucose and sodium malate (glu:mal) and 1:2 D-glucose and choline chloride (glu:chol), were mixed with 20\% w/w H\(_2\)O at 70 °C under magnetic stirring until a clear solution was obtained. The resulting stock solutions named 80\% NADES contained the following concentrations glu:tar (3.0 M D-glucose and 3.0 M dipotassium tartrate), glu:mal (3.8 M D-glucose and 3.8 M sodium malate), glu:chol (2.1 M D-glucose and 4.2 M choline chloride), with glycerol (8.7 M glycerol) as a control.

4.3. Growth of etiolated sorghum seedlings and preparation of microsomal fractions

Microsomes were prepared from Sorghum bicolor (L.) Moench (Poaceae) as described elsewhere (Halkier and Møller, 1989). Briefly, seeds of S. bicolor BTx-623 (Seedtek Pty Ltd, Toowoomba, Australia) were germinated between two layers of gauze for 3–4 d at 28 °C in the dark. Seedlings were harvested with a razor blade, avoiding collecting the seed coats. Seedlings were homogenized using a mortar and pestle in 2 × (w/v) homogenization buffer (250 mM sucrose, 100 mM Tris (pH 7.9), 50 mM NaCl, 2 mM EDTA, 2 mM DTT) and 10\% (w/w) of polyvinylpyrrolidone (PVPP). The homogenate was filtered through two layers of 22 μm nylon cloth, following which cell debris were removed by centrifugation (10,000 g, 4 °C, 10 min). The microsomal membranes were isolated from the supernatant obtained by ultracentrifugation (150,000 g, 4 °C, 1 h) and resuspended in resuspension buffer (50 mM Tris (pH 7.9), 100 mM NaCl, 2 mM DTT, DTT added just before use) using a marten hair soft brush and homogenized using a Potter-Elvehjem homogenizer.

4.4. Expression and purification of CYP79A1, CYP71E1, POR2B and UGT85B1

CYP79A1 and CYP71E1 were expressed in the yeast (Saccharomyces cerevisiae) strain BY4741 and purified as described elsewhere (Laursen et al., 2013, 2016; Pompon et al., 1996). POR2B was expressed in Escherichia coli cell strain BL21 (DE3) and purified as described elsewhere (Laursen et al., 2016; Wådsäter et al., 2012). UGT85B1 was expressed as a C-terminal fusion protein with green fluorescent protein (GFP) interspersed with a tobacco etch virus (TEV) protease cleavage site and including a His-tag as described elsewhere (Laursen et al., 2016).

4.5. Preparation of proteoliposomes

Native sorghum lipids were extracted with chloroform and methanol directly from microsomes prepared from etiolated sorghum seedlings (Bligh and Dyer, 1959). The concentration of phospholipids was determined by the method of Rouser et al. (1966). Liposomes were prepared with CYP79A1, CYP71E1 and POR2B reconstituted in native sorghum lipids as follows; Phospholipids (1000 nmol) were solubilized in buffer (50 mM Tris-HCl, 50 mM NaCl, 50 mM cholate, pH 7.5) and mixed with 1.3 nmol CYP79A1, 4 nmol CYP71E1 and 4 nmol POR2B in a total volume of 1000 μL and incubated for 1 h at 4 °C. Detergent was removed by addition of an equal volume of Bio-Beads SM-2 adsorbent (Biorad, Denmark) and incubation for 3 h at 4 °C. Bio-Beads were removed by centrifugation (1000 g for 20 min) and the supernatant was collected for further studies.

POR2B proteoliposomes were prepared in the same proportions as described above except without CYP79A1 and CYP71E1 and using 25\% DLPG/75\% DLPC (DLPG, 1,2-dilauroyl-sn-glycero-3-phosphoglycerol; DLPC, 1,2-dilauroyl-sn-glycero-3-phosphocholine (Avanti Polar lipids, USA).

4.6. Setups for enzyme activity and stability assays

4.6.1. NADES optimum on sorghum microsomes

The conversion of tyrosine catalyzed by the membrane-anchored POR2B, CYP79A1 and CYP71E1 to \( p \)-hydroxymandelonitrile was assayed in the presence of 0, 4, 8, 12, 16, 20, 24, 28, 36, 44 and 48\% NADES or glycerol. Assay conditions are described below.

4.6.2. Time course on proteoliposomes

Proteoliposomes containing POR2B (4.0 μM), CYP79A1 (1.3 μM) and CYP71E1 (4.0 μM) as described above and UGT85B1 (18.2 μM) were suspended in 10 vol buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.5), glycerol (28\% H\(_2\)O) or NADES (28\% H\(_2\)O). Samples were prepared in triplicates and stored at RT. For each time point, 10 μL was diluted 6× prior to activity assay (12% final concentration of glycerol and NADES).

4.6.3. Heat treatment on proteoliposomes

Samples were solubilized in buffer (100 mM NaCl, 50 mM Tris-HCl, 7.5), glycerol (28\% H\(_2\)O) or NADES (28\% H\(_2\)O)}.
pH 7.5), glycerol (28% H2O) or NADES (28% H2O) in a volume of 100 μl before heat treatment for 30 min at either 30 °C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C and 90 °C followed by cooling on ice for 30 min prior to activity assay.

4.7. Activity assays

Microsomes, proteoliposomes and recombinant UGT85B1 were incubated in 72% NADES for all experiments related to activity recovery, time course and heat treatment. Samples were diluted 6 × in buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.5) in the assay reactions.

4.7.1. Cytochrome P450 oxidoreductase, POR

Reductase activity was measured using cytochrome c (cyt c) as electron acceptor at different concentrations of NADES (containing 100-28% H2O) in buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.5). The assay mixtures (total volume: 1 ml) contained 0.12 pmol POR protein in 25% DLPG/75% DLPC liposomes, 1 mM NADPH and 50 μM cyt c. Reduction of cyt c was monitored at 550 nm using a PerkinElmer Lambda 650 UV/Vis spectrophotometer (extinction coefficient = 21.2 mM⁻¹ cm⁻¹). The slope of the curve in the linear region was used to determine the catalytic activity (Guengerich et al., 2009). For determination of reductase activity after heat treatment, POR samples were diluted to 6 × corresponding to a final concentration of 88% H2O in both NADES or glycerol samples prior to performing the activity assay.

4.7.2. Sorghum microsomes and recombinant cytochromes P450 enzymes (CYPs)

CYP activity was measured in assay mixtures (total volume: 30 μl) containing either microsomes (1.5 μg/μl total protein) or CYP79A1 (0.22 pmol) and CYP71E1 (0.67 pmol) in sorghum lipid proteoliposomes, 17 μM [UL-¹⁴C]-L-tyrosine (specific activity: 391 μCi/μmol, Larson & Fine Chemical AB, Malmö, Sweden), 100 μM L-tyrosine, 1 mM NADPH, 100 mM NaCl and 50 mM Tris-HCl (pH 7.5). Incubation (30 °C, 300 rpm, 30 min) was terminated by direct application of aliquots (10 μl) onto TLC plates (Silica gel 60 F₂₅₄, Merck). Substrates and intermediates were separated using a mobile phase of toluene: ethyl acetate: methanol: water (40:30:12:10 by volume). Authentic standards (40 pmol) were spotted on the TLC plates and visualized by UV luminescence. Radiolabeled reaction products were visualized by O/N exposure of the TLC plates to Phosphoimager screens (Molecular Dynamics) and the use of a standard curve of 5–100 pmol UDP-[¹⁴C]-L-tyrosine. For NADES optimum concentration determination ranging from 0.2% to 83.3 % of H2O in the assay were performed as described above and addition of various concentrations of NADES i.e. 0, 4, 8, 12, 16, 20, 40, 60 and 72% NADES.

4.7.3. UDP-glucosyltransferase (UGT)

UGT85B1 catalytic activity was measured in assay mixtures (total volume: 30 μl) containing recombinant UGT85B1 (1.2 pmol), 3.33 μM UDP-[UL-¹⁴C]-L-glucose (specific activity: 250 μCi/μl, PerkinElmer), 83.3 μM unlabeled UDP, 10 mM p-hydroxymandelonitrile (TCI Europe N.V.), 100 mM NaCl and 50 mM Tris-HCl (pH 7.5). Incubation (30 °C, 300 rpm, 30 min) was terminated by direct application of aliquots (20 μl) onto TLC plates (Silica gel 60 F₂₅₄, Merck). Substrates and intermediates were separated using a mobile phase of freshly mixed ethyl acetate: acetone: dichloromethane: methanol: water (40:30:12:10:8 by volume). For determination of the migration of the dhurrin, an authentic standard (40 pmol) was spotted on the TLC plates and visualized by UV luminescence. Radiolabeled reaction products were visualized by O/N exposure of the TLC plates to Phosphoimager screens (Molecular Dynamics) and developed using a Typhoon Trio™ and Typhoon scanner control software version 5.0. Product formation was quantified using the software ImageQuant TL ver. 7.0 and the use of a standard curve of 5–100 pmol UDP-[¹⁴C]-L-glucose.

Author contribution

C.K, B.L.M and T.L. designed the research project; C.K, K.B, K.M.V. and T.L. performed in vitro experiments; M.S.M. performed chemical synthesis of dhurrin and non-commercial intermediates; H.T.S., D.P.D, B.L.M. and T.L. initiated and supervised the research; C.K, D.P.D, B.L.M. and T.L. wrote the manuscript. All authors approved the final version of the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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