Cryobiology 68 (2014) 436–445

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Preservation of high phenylalanine ammonia lyase activities in roots of Japanese Striped corn: A potential oral therapeutic to treat phenylketonuria ☆

Arturo López-Villalobos [a], Joost Lücker [a], Ana Angela López-Quiróz [b], Edward C. Yeung [b], Kristoffer Palma [a], Allison R. Kermode [a,*]

[a] Department of Biological Sciences, 8888 University Dr., Simon Fraser University, Burnaby, British Columbia V5A 1S6, Canada
[b] Department of Biological Sciences, University of Calgary, 2500 University Drive N.W., Calgary, Alberta T2N 1N4, Canada

ARTICLE INFO

Article history:
Received 10 January 2014
Accepted 11 March 2014
Available online 18 March 2014

Keywords:
Phenylalanine ammonia lyase (PAL)
Oral enzyme therapeutic
Phenylketonuria (PKU)
Freezing preservation
Seedling roots
Japanese Striped corn
Oral therapeutic
Enzyme substitution therapy
Simulated digestion

ABSTRACT

Phenylketonuria (PKU) is an inherited metabolic disorder caused by deficient phenylalanine hydroxylase (PAH) activity, the enzyme responsible for the disposal of excess amounts of the essential amino acid phenylalanine (Phe). Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) has potential to serve as an enzyme substitution therapy for this human genetic disease. Using 7-day-old Japanese Striped corn seedlings (Japonica Striped maize, *Zea mays* L. cv. japonica) that contain high activities of PAL, we investigated a number of methods to preserve the roots as an intact food and for long-term storage. The cryoprotectant effects of maple syrup and other edible sugars (mono- and oligosaccharides) were evaluated. Following thawing, the preserved roots were then examined to determine whether the rigid plant cell walls could protect the PAL enzyme from proteolysis during simulated (*in vitro*) digestion comprised of gastric and intestinal phases. While several treatments led to retention of PAL activity during freezing, upon thawing and *in vitro* digestion, root tissues that had been previously frozen in the presence of maple syrup exhibited the highest residual PAL activities (~50% of the initial enzyme activity), in marked contrast to all of the treatments using other edible sugars. The structural integrity of the root cells, and the stability of the functional PAL tetramer were also preserved with the maple syrup protocol. These results have significance for the formulation of oral enzyme/protein therapeutics. When plant tissues are adequately preserved, the rigid cell walls constitute a protective barrier even under harsh (e.g. gastrointestinal-like) conditions.

Introduction

Phenylketonuria (PKU) is an inherited metabolic disorder caused by deficient phenylalanine hydroxylase (PAH; EC 1.14.16.1) activity, the enzyme responsible for the disposal of excess amounts of the essential amino acid phenylalanine (Phe), by its conversion to tyrosine. A Phe-restricted diet is still the current treatment for PKU patients and must be implemented immediately at birth to avoid the irreversible mental retardation and behavioral problems caused by Phe over-accumulation. Moreover, strict dietary compliance for the lifetime of the individual is required as ill effects occur even after stopping the diet in adulthood [14,25]. The challenges of adherence to the diet are not insignificant as individuals must ingest bland semi-synthetic liquid formulas (comprised of Phe-free amino acid mixtures, minerals, vitamins, and other nutrients) and further must avoid all of the normal protein-rich foods (e.g. meat, milk and grains) [6].

In principle, enzyme replacement with PAH should constitute a viable therapy for PKU; however this strategy has largely failed due to the enzyme's instability and requirement for cofactors. Thus enzyme replacement therapy with intact PAH and the full set of enzymes needed to maintain its co-factor BH_4 (6-R-L-erythro-5,6,7,8-tetrahydrobiopterin) is a daunting prospect that is not likely to be achieved in the short term. Another treatment involves dietary administration of the co-factor BH_4; however, patient response is

☆ *Statement of funding*: This work was funded by a Natural Sciences and Engineering Research Council of Canada - Canadian Institutes for Health Research (NSERC-CIHR) Collaborative Health Research Project grant (CHRPJ/385939-2010). A.R.K. is a recipient of a Michael Smith Foundation for Health Research Senior Scholar Award (No. CI-SSH-01915[07-1]).

* Corresponding author.
E-mail addresses: artlopezvillalobos@hotmail.com (A. López-Villalobos), jlucker@gmail.com (J. Lücker), aalopezq@ucalgary.ca (A.A. López-Quiróz), yeung@ucalgary.ca (E.C. Yeung), palma@neb.com (K. Palma), kermode@sfu.ca (A.R. Kermode).

http://dx.doi.org/10.1016/j.cryobiol.2014.03.003
0011-2240/

dependent on the particular *PAH* allele and its effect on PAH enzyme integrity, with the greatest response in milder non-PKU hyperphenylalanemia patients [4,39,43]. Enzyme substitution therapy, with phenylalanine ammonia lyase (PAL, EC 4.3.1.5), a Phe-degrading enzyme of plants, yeast and bacteria, currently shows the most promise for PKU treatment [12,13,31,36]. PAL requires no cofactor; it can act as a surrogate to the deficient PAH and convert the excess systemic Phe to *trans*-cinnamic acid and metabolically insignificant levels of ammonia [17]. *Trans*-cinnamate is a harmless product that is converted in the liver to benzoic acid and is then excreted as hippurate in urine [38], along with small amounts of cinnamate and benzoic acid [35]. Both pharmacological and physiological principles of therapeutic PAL efficacy have been confirmed by examining the efficacy of a recombinant yeast PAL given orally or by injection to the PKU mouse models [36]. Chemically and genetically engineered PAL formulations led to long-term reversal of hyperphenylalanemia [33]. However, there are still issues with the present formulations, and an injectable pegylated form of PAL (PEG-PAL) pursued by Biomarin Pharmaceutical Inc. has not yet been approved by the FDA for PKU treatment. Oral delivery of PAL is a viable option; the effectiveness of this mode of delivery of enzyme depends in part on the extensive enterorecirculation of amino acids between the body and the intestine. An orally delivered PAL would aid in the depletion of Phe in the intestine [9], and would further reduce Phe in all body pools [10] since amino acids are in equilibrium between the various compartments of body fluids and ultimately in equilibrium with the intestinal lumen. Yet oral formulations based on the naked isolated PAL enzyme are still ineffective despite many efforts at optimization, mainly due to the short lifespan of active recombinant PAL enzyme [34]. These studies have demonstrated that enzyme formulations comprised of protease-protected variants require a long period of contact with the substrate during passage through the small and large intestine. In addition, low specific activity means that a large amount of the recombinant PAL formulation is required to lower plasma Phe in PKU.

In order to bypass some of these appreciable hurdles associated with formulating an oral therapeutic based on purified PAL, we have pursued the endogenous PAL enzymes found in edible vascular plants [15,21,22], in which the enzyme serves as a key entry point in the phenylpropanoid pathway that produces many secondary metabolites such as flavonoids, anthocyanins, lignins, phytoalexins and other benzoic acid derivatives [37]. A survey of edible cereal sprouts was carried out in order to identify the varieties with intrinsically high PAL activities, and identified an ornamental variety, Japanese Striped corn (Japonica Striped maize, *Zea mays* L. cv. japonica), whose seedlings exhibit remarkably high levels of PAL specific activity in anthocyanin-containing root tissues [22]. To pursue the development of an edible oral therapeutic capable of lowering blood Phe levels of hyperphenylalanemic individuals, the final product must be preserved and protected. Importantly, PAL activity must be largely retained in a product that is stable and able to be stored using a food preservation method. Toward this, we previously determined that the PAL enzymes of Japanese Striped corn root tissues are robust; roots retain 90% of their PAL activity after freeze-drying and 50% activity after freeze-drying and subsequent 15-week storage at 4 °C. However, preservation methods need to additionally ensure that the integrity of the plant cell wall/membrane is not weakened, as this would lead to pronounced degradation of PAL during transit through the gastrointestinal (GI) tract.

In the present study, our major objectives were three-fold: (1) To investigate a number of methods, including freeze-drying, dehydration and freezing, both alone and in combination with various edible sugars, simple and complex sugar-mixtures, and oligosaccharides to preserve the roots as an intact food, and retain PAL enzyme activity. (2) To determine if the preservation methods also protect the roots and PAL activities during *in vitro* (simulated) digestion and (3) For the best preservation method determine the resilience of the root cells and the stability of the PAL protein in extracts.

We identified a suitable preservation method based on complex constituents afforded by the natural product maple syrup that maintains integrity of the Japanese Striped corn root cells, and preserves PAL activity even upon challenge with simulated digestion conditions. The preservation method will enable us to store sufficient quantities of root tissues for future studies to evaluate efficacy and ultimately undertake clinical studies of the oral therapeutic for PKU and related diseases. Finally, the method could be applied to the bio-encapsulation of other pharmaceutical enzymes intended for oral administration to treat human diseases.

Materials and methods

Plant materials and culture conditions

Seeds of Japanese Striped corn (*Zea mays* L. cv. Japonica Striped), Blue Jade, Indian Blue, 2-inch strawberry popcorn, Morado Black, Bloody Butcher, Blue Shaman and Ornamental Popcorn were obtained from Seed Savers Exchange (Decorah, IA, USA), and germinated to generate 3- to 12-day old seedlings. Under our growth conditions, the seeds germinated by day 2 after imbibition; seedlings used for the various preservation studies were 7 days old (i.e. 7 days after imbibition; DAI) as this stage is associated with high PAL specific activities in the roots. Briefly, seeds were surface disinfected in chlorine gas contained in a desiccator for 3 h. Following extensive rinsing in sterile distilled water, seeds were further disinfected for 2 min in 50% commercial bleach (Clorox® Brampton, On, Canada) (2.625% hypochlorite) adjusted to pH 7.5. After rinsing five times in sterile distilled water, seeds were left for 24 h in sterile distilled water. Seeds were then transferred to sterilized 130-ml culture tubes (Corning Inc., Corning, NY, USA) containing moistened Kimpak (Seedburo Equipment Co., Chicago, IL, USA) and maintained in a growth chamber at 22 °C, with a 16-h photoperiod and a light intensity of approximately 70 μmol m^{-2} s^{-1}. Roots of the 7-day-old seedlings were harvested and used immediately for analyses or treated as described below.

Freeze drying of corn roots

Freeze-drying of seedling roots was performed as described [21]. In cases where the effect of sucrose pre-treatment was evaluated, intact seedlings were first vacuum-infiltrated with sucrose solutions (30% and 60%) for 4 h. The roots were then excised from the seedlings, frozen for 24 h at −20 °C, and subsequently freeze-dried overnight.

General procedure for combined preservation treatments

Various preservation treatments were conducted on intact 7-day-old seedlings to assess their effectiveness in retaining the PAL activity of the excised roots stored at −20 °C. In some cases, retention of PAL activity during simulated *in vitro* gastric (or gastro-intestinal) digestion was also evaluated. A basic protocol was followed, unless otherwise detailed. First, a 3-h pre-treatment of seedlings was performed in which the intact corn seedlings were incubated in culture tubes containing 20 ml of the 'cryoprotectant' solution (solutions comprised of a simple sugar, sugar mixture, oligosaccharide, or maple syrup; see below). Subsequently, approximately 400 mg roots (generally the roots of 3 seedlings combined) were excised from the treated seedlings and transferred into a 15-ml polypropylene tube containing 5 ml of the same cryoprotectant solution as used in the pre-treatment. The tubes with the roots completely immersed in the cryoprotectant solution were stored