Development of phytase-expressing chlamydomonas reinhardtii for monogastric animal nutrition

Fernanda Erpel†, Franko Restovic† and Patricio Arce-Johnson*

Abstract

Background: In plant-derived animal feedstuffs, nearly 80% of the total phosphorus content is stored as phytate. However, phytate is poorly digested by monogastric animals such as poultry, swine and fish, as they lack the hydrolytic enzyme phytase; hence it is regarded as a nutritionally inactive compound from a phosphate bioavailability point of view. In addition, it also chelates important dietary minerals and essential amino acids. Therefore, dietary supplementation with bioavailable phosphate and exogenous phytases are required to achieve optimal animal growth. In order to simplify the obtaining and application processes, we developed a phytase expressing cell-wall deficient Chlamydomonas reinhardtii strain.

Results: In this work, we developed a transgenic microalgae expressing a fungal phytase to be used as a food supplement for monogastric animals. A codon optimized Aspergillus niger PhyA E228K phytase (mE228K) with improved performance at pH 3.5 was transformed into the plastid genome of Chlamydomonas reinhardtii in order to achieve optimal expression. We engineered a plastid-specific construction harboring the mE228K gene, which allowed us to obtain high expression level lines with measurable in vitro phytase activity. Both wild-type and cell-wall deficient strains were selected, as the latter is a suitable model for animal digestion. The enzymatic activity of the mE228K expressing lines were approximately 5 phytase units per gram of dry biomass at pH 3.5 and 37 °C, similar to physiological conditions and economically competitive for use in commercial activities.

Conclusions: A reference basis for the future biotechnological application of microalgae is provided in this work. A cell-wall deficient transgenic microalgae with phytase activity at gastrointestinal pH and temperature and suitable for pellet formation was developed. Moreover, the associated microalgae biomass costs of this strain would be between US$5 and US$60 per ton of feedstuff, similar to the US$2 per ton of feedstuffs of commercially available phytases. Our data provide evidence of phytate-hydrolyzing microalgae biomass for use as a food additive without the need for protein purification.

Keywords: Microalgae, Phytase, Animal nutrition


(myo-inositol hexakisphosphate phosphohydrolases) on the other hand, are part of a phosphatase family that catalyzes the sequential dephosphorylation of phytate [7]. They are divided into two groups, according to phylogenetic analysis and biochemical properties: alkaline phytases, that have a strong preference for calcium-binding phytate; and the histidine acid phytases (HAP), which use mostly free phytate as substrate and show enzymatic activity at acid pH (between 2.5 and 6) and temperatures over 40 °C [8]. The application of phytases into the diet of monogastric animals has been adopted worldwide, not only with benefits for animal growth, but also for the environment. The use of HAPs has been directly correlated with a higher phosphate, mineral and essential amino acid bioavailability [3, 4]. Moreover, replacement of exogenous dicalcium phosphate by phytases, diminishes the phosphate concentration in swine excretions by 30–60 % [9]. An ideal phytase model should 1) be able to effectively hydrolyze phytate in order to obtain sufficient inorganic phosphate to supply the animal requirements, 2) be able to resist high temperatures in order to be incorporated into food pellets, and 3) have low production costs. Kim, Mullaney [10] developed single and multiple mutants of the Aspergillus niger PHYA gene, resulting in a single mutant (E228K) with a shifted optimum pH (from 5.5 to 3.8) and 266 % greater hydrolysis of phytate at pH 3.5 compared to the wild-type version, the same pH as found in the gastrointestinal system of monogastric animals.

On the other hand, microalgae have generated an enormous industrial interest as model systems for the production of added-value molecules. Among these, Chlamydomonas reinhardii is the most recognized microalga due to its suitability for genetic transformation and scalability; moreover, various examples of recombinant protein expression are available [11–14]. Improved recombinant protein production has been achieved by targeting via homologous recombination, specific plastid regions in order to obtain high gene expression, bypassing the known nuclear gene silencing that affects this organism [11, 12]. Several mutant strains of C. reinhardii are available, among them the cell-wall deficient strain cw15, which is an excellent model for easily digestible microalgae [15]. Additionally, these organisms are an excellent source of carbohydrates, proteins and several nutrients, and have been accepted as Generally Recognized As Safe (GRAS), ensuring their use in the food and medical industries [16]. From an economic standpoint, microalgae are low cost production bio-factories; estimated costs based on antibody production in mammal, plant and microalgae systems are US$150, US$0.005 and US$0.002, respectively [17]. Taken together, microalgae are thus excellent model organisms for use in animal feeds due to the ecological, economical and practical advantages. For these reasons, we expressed an optimized version of the PHYA E228K gene (hereafter named mE228K) in both wild-type and cell-wall deficient cw15 C. reinhardtii strains. Cell wall mutants could be useful in applications where cell disruption is needed, as for example, in animal nutrition. Phytase produced by microalgae effectively hydrolyzes phytate and should be able to resist high pelleting-like temperatures.

Results & discussion

The potential of microalgae for producing high-value molecules, and their use in economically important activities as an oral administration vehicle have already been demonstrated [18]. C. reinhardtii has been extensively used as a model protein factory, and here we show how a cell-wall mutant (cw15) could be used in the administration of phytase into animal feedstuffs. We chose an improved version of the PhyA gene of Aspergillus niger, with a higher activity at pH 3.5, as found in the gastrointestinal system of monogastric animals [10].

Codon optimization of the PHYA E228K gene

We chose the PhyA E228K mutant, developed by Kim, Mullaney [10], as the phytase gene to express in C. reinhardtii. The wild-type version of PhyA has a double activity-peak pH profile, with two marked peaks at pH 2.5 and 5.5 [19]. However, the E228K mutant shifts the optimal pH to 3.8 and greatly increases phytate hydrolysis at pH 3.5, which is the typical pH of the digestive tract of monogastric animals [10]. In order to obtain an increased expression in C. reinhardtii, we performed codon optimization of the E228K mutant nucleotide sequence. A C. reinhardtii codon usage table was obtained from Nakamura, Gojobori [20], and this information was used in the OPTIMIZER webpage, where the optimization of the desired sequence was undertaken [21, 22]. It is worth noting that only the mature protein sequence was used, devoid of the extracellular destination N-terminal peptide, in order to trap the enzymatic activity within the intracellular space of the transformed microalgae. The resulting optimized gene (mE228K) was synthesized and cloned into a SmaI-digested pBluescript II SK(-) derivative lacking its multiple cloning sites (EPOCH Life Science, http://www.epochlifescience.com/). Further analysis of the optimized gene showed that more than half of the codons were optimized (51.56 %), whereas the sequences have a 78.1 % of nucleotide identity, and maintain 100 % amino acid identity. The detailed sequence information is shown in Fig. 1.

Determination of transformation pressures

In order to optimize wild-type (UTEX-90) and cw15 transformation, we subjected both strains to different
shooting pressures of between 250 and 600 psi with an empty p3HB-Kan vector. Kanamycin resistant colonies were counted after incubation for 7 days in the presence of the antibiotic. As expected, more UTEX-90 colonies appeared at higher pressures (between 400 and 600 psi), whereas the cell-wall deficient strain was more efficiently-transformed at a lower pressure (near 350 psi; Fig. 2). Following this result, we determined 500 and 350 psi as the optimal shooting pressures for UTEX-90 and cw15 strains, respectively.

Cloning into the p3HB-Kan vector and transformation of *Chlamydomonas reinhardtii*

The synthesized gene (mE228K) was cloned into the p3HB-Kan-vector between the NdeI and XbaI sites [12]. The resulting vector (p3HB-Kan-mE228K) contains two regions of inverted repeats of the *C. reinhardtii* chloroplast genome (for homologous recombination) flanking a construction bearing the PSBD promoter/5′UTR, the optimized mE228K gene and the PSBA3′UTR region, in addition to the APHA6 gene flanked by the ATPA promoter/5′UTR and the RBCL3′UTR, for kanamycin resistance (Fig. 3a). In order to transform *C. reinhardtii*, the p3HB-Kan-mE228K plasmid was coated onto nanogold particles, and then delivered into cells of UTEX-90 and cw15 strains plated in TAP-Agar plaques using a biolistic approach with shooting pressures of 500 and 350 psi, respectively. After two weeks of incubation at 16 h light/8 h dark and 24 °C in the presence of kanamycin, we obtained 65 resistant colonies, which were subsequently inoculated into 1 ml of liquid TAP medium without antibiotics for recovery. From these colonies, 30 were classified as stable transformants as determined by amplification of a vector region comprising part of the
promoter and the \textit{mE228K} gene, and at least two further sub-cultures in selective media (Fig. 3b).

**Expression of the \textit{mE228K} transgene in transformed UTEX-90 and \textit{cw15} strains**

To determine if kanamycin resistant colonies were expressing the \textit{mE228K} transcript, we selected the four best-growing UTEX-90 (L18, L19, L20 and L23) and \textit{cw15} (L2, L3, L5 and L31) transformed lines (data not shown) and analyzed them by RT-qPCR using \textit{mE228K} specific primers. All transformed lines expressed the phytase gene between 75 and 275-fold compared to non-transformed controls (Fig. 4).

**Phytase activity determination**

A functional in vitro assay was performed in order to assess the enzymatic activity of the transformed microalgae lines. This method consisted in the detection of the released inorganic phosphate from exogenous phytic acid, which is measured colorimetrically. The total absorbance detected at 400 nm is directly proportional to the liberated inorganic phosphate. The microalgae lines
with significant \textit{mE228K} expression were lyophilized, and the resulting dry biomass was resuspended with phytate and assayed for phytase activity for 30 min at 37 °C at pH 3.5. We obtained three lines with significant phytase activity. Of these lines, two corresponded to \textit{cw15} lines (L5 and L31) and the remaining to a UTEX-90 line (L23; Fig. 5).

Surprisingly, highest phytase activity (L5) did not correlate with higher gene expression, as transcript levels of L23 and L31 lines were approximately 2.8 and 3.6 fold higher than L5, respectively. Focusing on L5, the phytase activity of this line as determined at 37 °C and pH 3.5, was 5 units per gram of lyophilisate (μmols of free phosphate per 30 min * 0.1 mL of homogenate * 50 mg of lyophilisate dissolved in 1 mL of homogenate). Commercial product recommendations for phytase use in pig and chick feed are near 250–500 units per kg of feedstuffs (Natuphos®; taking into consideration that commercial units are determined at pH 4.5, and not 3.5, the actual digestive system pH of monogastric animals). This means that 50–100 g of lyophilized microalgae would be sufficient for every kg of feedstuffs (Natuphos®; taking into consideration that commercial units are determined at pH 4.5, and not 3.5, the actual digestive system pH of monogastric animals). This means that 50–100 g of lyophilized microalgae would be sufficient for every kg of feedstuffs (without considering the additional nutrients that microalgae biomass possess). Considering the costs of microalgae biomass production, estimates indicate that they would range between $50 and $600 per ton, with conservative yields of at least 20 g dry weight m$^{-2}$ day$^{-1}$ [18, 23]. Altogether, the associated microalgae biomass costs would be between US$5 and US$60 per ton of feedstuff, or even less at higher yields. These costs are relatively similar to the ones associated with phytase use in livestock feeding, which are near US$2 per ton of feedstuff [6]. Furthermore, \textit{C. reinhardtii} (and microalgae in general) has high nutritional value, with lipid, carbohydrates and total
protein contents close to 21, 17 and 48 % of its dry weight, respectively [24], meaning that the inclusion of microalgae biomass in feedstuffs could reduce the need to supplement feeds with other amino acids and necessary nutrients. In addition, C. reinhardtii is classified as GRAS, making this organism a suitable model for protein expression and commercialization [25, 26]. The utilization of the cell wall deficient strain cw15 should additionally provide a more suitable substrate for animal digestion, which could mean a higher phytase bioavailability in the gastrointestinal system, without the need for protein purification.

Conclusions
Phytase-expressing C. reinhardtii strains were developed to tackle the nutritional problems regarding phosphorus deficiency and general animal nutrition. The use of cell-wall deficient strains should become a viable option for delivering specific enzymatic activities in animal nutrition. Animal experiments are needed to confirm the in vivo phytase activity and to assess the safety and nutritional benefits associated with microalgae consumption.

Methods
Microalgae culture
The Chlamydomonas reinhardtii UTEX-90 (wild-type) and cw15 strains were obtained from the Microalgae Culture Laboratory from the Universidad de Concepción and the Chlamydomonas Resource Center (http://www.chlamycollection.org/products/strains/), respectively. C. reinhardtii were grown in Tris-acetate-phosphate medium (TAP) at 25 °C, with a 16 h light/8 h dark photo-period in an orbital shaker at 180 rpm with 1000 lux.

Cloning and obtaining of transformed microalgae
The p3HB-Kan vector was a kind gift of Dr. Beth Rasala and Dr. Stephen Mayfield [11, 12]. Codon optimization of the E228K gene was performed using the C. reinhardtii codon database (http://www.kazusa.or.jp/codon/) described by Nakamura, Gojobori [20]. The resulting optimized PHYA gene (mE228K) was synthesised in the pBSK GS52811 vector containing an ampicillin resistance gene between the Ndel and XbaI restriction sites (Epoch Life Science). The mE228K gene was digested and subsequently cloned into p3HB-Kan, harboring the Ndel and XbaI sites. After cloning into p3HB-Kan, the insertion was confirmed by DNA sequencing.

Microalgae transformation by biolistics was performed as described in Rasala, Muto [11]. Briefly, UTEX-90 and cw15 strains were cultivated in liquid TAP medium until the exponential growth stage (approximately 10⁶ cells/mL; DO₂: 50–60% 0.4). Later, cells were spun down, resuspended in TAP medium at a concentration of 10⁹ cells/mL, and 10 µL aliquots were cultivated in TAP-agar plates with 50 µg/mL kanamycin. Afterwards, plated cells were subjected to transformation using the Helios® Gene Gun (Bio-Rad, CA) with shooting pressures between 250 and 600 psi. The p3HB-Kan-mE228K vector was coated onto gold particles following manufacturer’s instructions. After transformation, cells were incubated for 7–10 days until growth of kanamycin-resistant colonies. The resistant colonies were inoculated into 1 mL of liquid TAP medium without antibiotic for recovery. Stable transformants were determined by Taq polymerase (Invitrogen) amplification of a vector region comprising part of the promoter and the mE228K gene (primers: 5′-TTGTTTTTTTTATTTTG GAGATACACGCC-3′ and 5′-TCCTCGATCGGGGCGC TGTA-3′), and growth in at least two further sub-cultures in selective media.

Transcript expression determination
Real time quantitative PCR (RT-qPCR) was performed in order to determine mE228K gene expression in transformed microalgae. Briefly, microalgae were homogenized in a Precellys® 24 (Berlin Technologies) and RNA was obtained using the TRIzol® reagent (Invitrogen). Then, 2 µg of RNA were treated with the Turbo DNA-Free™ kit (Ambion™) and cDNA was made with random primers and Superscript II® Reverse Transcriptase (Invitrogen) following the manufacturer’s protocol. Quantification of the mE228K transcripts was performed using the Mx3000p™ (Stratagene™) thermocycler and the SensiMix™ Plus SYBR kit (Quantace) following manufacturer’s procedure. The RBCL gene was used as an expression normalizer, as described in Rasala, Muto [12]. Primers used were as follow: 5′-CTTGCGAGG CCTGAAGAGT-3′ and 5′-CGAACAGGATGCTGATGATG-3′ for mE228K; 5′- CAGTTGCTTCAGGGCGTTAT-3′ and 5′-AATTACG TCGCCACCTTCCAC-3′ for RBCL.

Phytase activity assay
Wild-type and phytase expressing cells were grown in 400 mL of selective media until the exponential growth phase (approximately 10⁶ cells). Cultures were centrifuged, washed with 1 % glycerol and centrifuged again. The resulting pellets were frozen with liquid nitrogen, thawed and frozen again previous to lyophilization in a Freezezone 2.5 Benchtop Liter Lyophilizer (Labconco). The lyophilized whole-cell lysates (dry microalgae biomass) were used for phytase activity assays.

Phytase activity quantification was performed as described by Heinonen and Lahti [27] with modifications. Briefly, 25 mg of lyophilized microalgae were dissolved in 500 µL of 200 mM pH 3.5 sodium acetate – acetic acid buffer. In order to start the catalytic reaction, 100 µL of each sample were added to 500 µL of a 44.1 mM sodium phytate solution (in acetate buffer) previously incubated at 37 °C for 5 min. The mock was performed under the same conditions.
conditions without the lyophilized microalgae, and a positive control was carried out adding 100 µL of a commercial phytase solution (Natuphos®). After incubation for 30 min at 37 °C, 4 mL of CRS colorimetric solution (acetone, 5 N sulphuric acid and 5 % ammonium molybdate; 2:1:1 v/v/v) was added, and absorbance (400 nm) was measured. A standard curve of absorbance was made with phosphate (0.5–2.5 µmol). The enzymatic activity (units per mg of lyophilisate) was obtained as the units of released phosphate per duration of assay, volume and lyophilisate mass.

Availability of data and materials

The dataset supporting the conclusions of this article is available in the GenBank (National Center for Biotechnology Information) repository KT899873.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

FE and FR contributed to the conception of the study. FE carried out the cloning and obtained the transformed microalgae. FE and FR did the phytase assays of transformed lines. FR drafted the manuscript. FE and PAJ were involved in revising the manuscript critically for important intellectual content. PAJ gave final approval of the version to be published. All authors read and approved the final manuscript.

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