Phenylalanine Response in Rice Kernel Under Chitosan Nanoparticles Spraying

Mohammad Reza Ghalamboran (✉ m_ghalamboran@sbu.ac.ir)

Shahid Beheshti University <https://orcid.org/0000-0003-3865-8636>

Azar Kohnavard

Shahid Beheshti University

Seyedeh Batool Hassani

Shahid Beheshti University

Research

Keywords: Chitosan nanoparticles, Phenylketonuria, Phenylalanine, Rice

Posted Date: December 23rd, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-131962/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: Since phenylketonuria patients have a metabolic defect, should use specific foods that have low phenylalanine levels. These foods are usually produced by hydrolysis processes. The most important problems of the foods are unpleasant to taste and expensive. The main objective of this study was to investigate the possibility of reducing the amount of phenylalanine in rice kernel under spraying of chitosan nanoparticles. Treatments were different concentrations of chitosan nanoparticles spraying (0,500,1000,1500 μL^{-1}). The treatments effects were studied in a randomized complete block design with four replications. Experimental variables were phenylalanine, phenylalanine ammonia-lyase activity and total grain protein.

Results: The results showed that the total protein and phenylalanine level in rice kernel decreased under all concentrations of chitosan nanoparticles compared to the control, while the activity of phenylalanine ammonia-lyase was higher than that of the control. In this experiment, 1000 μL^{-1} of chitosan nanoparticles was found as the optimum concentration, since minimum phenylalanine level and maximum phenylalanine ammonia-lyase activity were affected by this concentration.

Conclusion: Our results indicated that chitosan nanoparticles spraying during the grain growth was able to reduce phenylalanine level in rice kernel.

Background

Phenylketonuria (PKU) is an autosomal recessive genetic disorder in humans that occurs due to mutations in phenylalanine hydroxylase genes and as a metabolic defect, disrupts the pathway of phenylalanine (Phe) metabolism. In fact, the synthesis and activity of the phenylalanine hydroxylase enzyme is reduced due to a genetic disorder and as a result, the Phe metabolism process will be incomplete. Then, the Phe level in the body fluids (such as blood plasma and urine) of patients is increased. As the level of Phe increases, its derivatives will increase as well as the amount of phenylpyruvate in body fluids. Increasing the amount of phenylpyruvate in the blood prevents the allosteric action of pyruvate decarboxylase in the brain. The production of tyrosine is a result of the effect of phenylalanine hydroxylase on the Phe, and if the activity of phenylalanine hydroxylase is reduced, then reduces tyrosine level [1, 2]. Decreased tyrosine level in body fluids of patient is the most important consequence of a metabolic defect, as tyrosine is a precursor to the production of neurotransmitters such as dopamine and serotonin [3]. Severe deficiencies of tyrosine, dopamine and serotonin compounds cause paralysis and disruption of the central nervous system in the patient. One way to treat the PKU patients is changing diets and using processed semi-medicated foods to prevent the rising and accumulation of the Phe level in body fluids.

Currently, the main strategy of food manufacturers for PKU patients is to hydrolyze food and then combine hydrolyzed foods with synthetic nutrients such as nutrients and vitamins [4]. This strategy has disadvantages such as high product prices, reduced foods quality during the hydrolysis process,

restrictions on the use of different types of foods, low shelf life, high spoilage and ultimately an unpleasant taste in food products. For example, manufacturers have to use large quantities of sweeteners to overcome the unpleasant taste of processed foods, which in turn increase problems for patients who, in addition to having the PKU.

However, despite these problems, the PKU patients require the Phe for their health and normal growth processes and should receive the Phe under the supervision of a physician and nutritionist. Therefore, the production of food for the PKU patients is complex and costly. As the majority of developing and underdeveloped countries cannot afford and pay all the costs of the PKU patients, and these countries have serious challenges in treating and caring for the PKU patients.

It is clear that other strategies should be used to help these patients to avoid the disadvantages of past methods. It means that, a safer and easier strategy could be devised to reduce naturally occurring the Phe level in food products. For example, through plant nutrition management and changes in plant growth management systems, so that their products naturally contain a small amount of the Phe. In fact, the present study was designed based on this strategy to determine whether the Phe level in grain, such as rice, could be reduced by the use of natural elicitors during grain growth.

In fact, rice has a high nutritional value, and it is one of the most important foods for the PKU patients. Based on proteomics research on rice leaves, roots and seeds, more than 2,500 types of protein belonging to approximately 11 functional classes have been identified [5]. The cytoplasmic and storage proteins of cereal grains differ greatly in the type and composition of amino acids, and these differences determine the qualitative of the grains. For example, in wheat grain storage proteins the levels of glutamic acid, proline and the Phe are higher than those of lysine, arginine, threonine and tryptophan, whereas in the grain metabolically active proteins, the levels of glutamic, proline and the Phe are much lower than that of lysine and arginine [6].

Thus, the effect of any limiting or modifying factor on amino acid synthesis pathways can affect the amino acid level and nutritional value of the grain[7][7][7]. Therefore, the capacity of limiting agents can be used to limit the biosynthetic pathway and reduce the production of the Phe amino acid in rice grain.

Previous studies have reported that the most important limiting factors of amino acids production in plants are functional disruption of regulators of amino acids biosynthesis pathways [8]. Regulatory factors include the nutrients, the favorable environmental conditions (e.g., temperature and pH) [9], and biochemical compounds (which are able to interfere with the amino acid synthesis pathway e.g., hormones and ROS) [10]. If necessary for cell survival, plant-related regulatory factors can play two opposing roles in the process of producing amino acids, by, stimulating or inhibiting. The occurrence of these roles depends on the physiological stages of cell growth, environmental conditions, nutritional conditions, the use of external elicitors, and damages caused by biological and non-biological stresses [11]. Consequently, the use of external elicitors to stimulate or limit the Phe synthesis pathway can be a reasonable strategy way to reduce the Phe level in the rice kernel. For example, an appropriate elicitor can

increase production and phenylalanine ammonia-lyase (PAL) activity during grain growth of rice, subsequently the Phe level can decrease in the kernel.

The PAL enzyme is main responsible for the metabolism of the Phe, and also it acts as a key enzyme in the phenylpropanoids synthesis pathway. The PAL enzyme converts the Phe from the primary metabolic source by removing non-oxidative ammonia (de-amination) to trans-cinnamic acid, then trans-cinnamic acid is converted to phenolic compounds. This means that through enzymatic activity on the Phe, then, the Phe level is reduced. The PAL enzyme belongs to the large family of ammonialyase or ammonia mutase and has multiple isoforms [12].

However, stimulating and to boost the synthesis and enhancing the PAL activity through an elicitor requires the design and construction of an effective and intelligent compound that not only enhances the synthesis and activity of the PAL, but also has the potential to compete with inhibitors of the PAL. Thus, advanced technologies are needed to make compounds that can be able trigger specific reactions in the amino acids biosynthesis pathway, as well as modify the product of the synthesis process.

Among the advanced technologies, nanotechnology is a powerful tool that can control and even modify the processes and metabolic activities of biological cells by producing nanoscale materials and controlling the properties of materials at the molecular and atomic level [13]. Production of nanoparticles from biocompatible materials like chitosan, is prioritized for use in the modification and improvement of biological systems.

Chitosan is a natural biopolymer derived from chitin, chitin is a major component of the cell wall structure of some fungi, shrimp and crab shells. Chitosan is synthesized by removing the acetate of the chitin through hydrolysis under alkaline conditions (sodium hydroxide) or through enzymatic hydrolysis in the presence of chitin deacetylase [14].

Chitosan is not only an antimicrobial agent but also an effective elicitor in the plant defense against pathogens. Recent research has shown that the use of chitin and chitosan in monocotyledon and dicotyledonous plants can induce defensive responses during invasion of pathogens. These responses include membrane depolarization, lignification and protein phosphorylation, activation of chitinase, phytoalexins biosynthesis and stimulation of defense mechanism-related gene expression [15] .

The favorable physiological responses of plants to chitosan-based nanoparticles are due to the change in particle size, meaning that its high surface-to-volume ratio is greater than that of bulk chitosan. Therefore, chitosan-based nanoparticles can be used to improve plant growth. The most important properties of chitosan nanoparticles include biocompatibility, biodegradability, high permeability, cost-effectiveness of production, non-toxicity, and excellent film forming ability as a suitable coating and carrier for drug delivery and antibacterial delivery [16]. The molecular structure of chitosan also has active functional groups O, H, NH₂, OH, and these groups provide the advantage of using chitosan for a variety of researches and industrial purposes [17]. Totally, chitosan nanoparticles have a larger number of

functional groups, due to their smaller size than the bulk particles, so they can participate effectively in the biochemical reactions processes, like the amino acids synthesis pathways.

The hypothesis of the present study was that if chitosan nanoparticles were used at the site and time of the Phe metabolic reactions in the grain, it could affect the Phe biosynthesis pathway and decrease the Phe level in the grain. Thus, the main objective of this study was to investigate the possibility of reducing the Phe level in rice kernel using chitosan nanoparticles. Therefore, the spraying application effects of different concentrations of chitosan nanoparticles at three stages of physiological growth of rice grain (spike onset, flowering onset, and milky onset of the grain) on experimental variables (amino acids level especially the Phe level and the PAL activity and total protein in kernel) were studied and examined.

Results

The analysis of variance results (Table 1) indicated that, the responses of the experimental variables were significantly influenced by the spraying application of chitosan nanoparticles during growth and development of rice grain (spike emergence, flowering initiation, and milking stage).

Table 1					
Mean square of experimental variables (total of essential amino acids, the Phe level, the PAL activity and total protein of rice kernel) under the influence of spraying application of chitosan nanoparticles.					
Source of variation	Degree Or freedom	The Phe level / mg g ⁻¹ protein	The PAL activity / μmol trans-cinnamic acid mg min ⁻¹	Total of essential amino acid† per rice kernel / mg g ⁻¹	Total protein of rice kernel / mg g ⁻¹
Chitosan nanoparticles	3	0.25**	2.1**	2.68**	0.5*
Error	9	0.001	0.42	0.58	0.06
*,**, Significantly different at 95% and 99% probability levels, respectively.					
†, Essential amino acids (histidine, valine, isoleucine, phenylalanine, tryptophan, leucine, threonine, lysine and methionine)					

The Phe response

The results showed that the Phe level was affected by spraying application of chitosan nanoparticles during the grain growth and development (Table 1). The minimum of the Phe level in the rice kernel was observed in 1000 μl L⁻¹ of chitosan nanoparticles as shown in the Fig. 1-A.

The PAL activity

The Results showed that the PAL activity was significantly affected by foliar application of chitosan nanoparticle concentrations (Table 1). The maximum of the PAL was observed in 1000 μL^{-1} of chitosan nanoparticles as shown in the Fig. 1-B.

The total protein of kernel

The total protein in rice kernel was affected by the spraying application of chitosan nanoparticles during growth and development of rice grain (Table 1). Two concentrations of the chitosan nanoparticles (1000 and 1500 μL^{-1}) significantly decreased the total protein level in the kernel (Fig. 1-C).

Essential amino acids responses

Analysis of variance (ANOVA: Table 1) was used to determine the influence of spraying application of chitosan nanoparticles concentration during the grain growth and development on amount of essential amino acids levels in the rice kernel. So, production levels comparisons of all essential amino acids were significantly different from control treatment, and also the means total weight of essential amino acids in grains treated with all concentrations of chitosan nanoparticles were lower than control treatment (Table 2).

Table 2

Mean comparisons (Duncan test) of essential amino acids level in the rice kernel (mg g^{-1}) by different concentration of chitosan nanoparticle spraying.

		Chitosan nanoparticles concentrations / $\mu\text{l L}^{-1}$		
Essential amino acids	Control	500	1000	1500
Histidine	0.429 a	0.331 b	0.421 a	0.193 c
Valine	0.000145 c	0.00041 b	0.037 a	0.0368 a
Isoleucine	0.0299 b	0.0308 b	0.163 a	0.168 a
Phenylalanine	0.175 a	0.168 a	0.0465 c	0.072 b
Tryptophan	0.00168 a	0.00152 ab	0.00135 bc	0.000699 d
Leucine	0.312 ab	0.352 a	0.214 c	0.177 cd
Threonine	0.0392 bc	0.0285 d	0.048 b	0.0689 a
lysine	0.036 b	0.0317 bc	0.0455 a	0.048 a
Methionine	0.813 a	0.198 b	0.0616 c	0.0633 c
Total weight of essential amino acids	1.84	1.14	1.04	0.83
Note: Alphabetical mismatch indicates significant difference at 99% probability level.				

Table 3

Mean comparisons (Duncan test) of amino acids levels of rice kernel (mg g^{-1}) by different concentration of chitosan nanoparticle spraying.

Amino acid	Chitosan nanoparticles concentrations / $\mu\text{l L}^{-1}$			
	Control	500	1000	1500
Asparagine	2.01 a	0.95 c	1.63 b	0.076 d
Methionine	0.813 a	0.198 b	0.0616 c	0.0633 c
Histidine	0.429 a	0.331 b	0.421 a	0.193 c
Tyrosine	0.319 b	0.133 c	0.48 a	0.499 a
Leucine	0.312 ab	0.352 a	0.214 c	0.177 cd
Serine	0.303 a	0.126 b	0.306 a	0.111 b
phenylalanine	0.175 a	0.168 a	0.0465 c	0.072 b
Aspartic	0.0777 a	0.051 b	0.0458 bc	0.0614 ab
Glutamine	0.0757 a	0.0562 b	0.0786 a	0.0396 bc
Arginine	0.0742 a	0.053 b	0.0465 bc	0.451 bc
Alanine	0.0463 b	0.0248 c	0.0558 a	0.227 c
Threonine	0.0392 bc	0.0285 d	0.048 b	0.0689 a
Glycine	0.0361 a	0.0199 b	0.0328 a	0.0347 a
Lysine	0.036 b	0.0317 bc	0.0455 a	0.048 a
Isoleucine	0.0299 b	0.0308 b	0.163 a	0.168 a
Tryptophan	0.00168 a	0.00152 ab	0.00135 bc	0.000699 d
Valine	0.000145 c	0.00041 b	0.037 a	0.0368 a
Total weight of amino acids	4.78	2.5	3.7	2.3
Note: Alphabetical mismatch indicates significant difference at 99% probability level.				

Comparisons results of the means total weight of amino acids produced in rice kernel by chitosan nanoparticle spraying showed that the mean total weight of amino acids produced, by $1000 \mu\text{l L}^{-1}$ concentration was higher than other chitosan concentrations (500 and $1500 \mu\text{l L}^{-1}$) (Tables 3). Although the means total weight of amino acids produced in the all concentrations were lower than the control

treatment, but the difference between 1000 μL^{-1} of chitosan nanoparticles with the control was lower than the other concentrations (Table 3), therefore the means total weight of amino acids produced by 1000 μL^{-1} of the chitosan nanoparticles was close to the control treatment.

However, the responses of amino acids produced in the rice kernel was not uniform between control treatment and 1000 μL^{-1} of chitosan nanoparticle. It means that by 1000 μL^{-1} of chitosan nanoparticle, some of amino acids increased (e.g.: Tyrosine, Alanine, Threonine, Lysine, Isoleucine and Valine), and others decreased (e.g.: Asparagine, Methionine, Leucine, Phenylalanine, Aspartic, Arginine, Glycine and Tryptophan) and the rest were similar to the control treatment (e.g.: Histidine, Serine and Glutamine). Among all of the concentrations, 1000 μL^{-1} of the chitosan nanoparticles spraying was optimum concentration in this experiment, because it has the maximum activity of the PAL and tyrosine level, and also minimum of the Phe level in the rice kernel.

Discussion

Based on the results of this experiment, using of chitosan nanoparticles spraying, during rice grain growth and development (spike onset, flowering onset and milky onset of grain), resulted in reduced total protein, total weight of amino acids, especially phenyl level in the rice kernel compared to control treatment.

Of the most important reasons for the metabolic response of rice grains to the effect of chitosan nanoparticle spraying, is the role and importance of the molecular structure of chitosan nanoparticles. Chitosan nanoparticles were able to interfere with amino acid synthesis pathways due to their active functional groups (OH, H, NH₂, O) and altered the enzymatic processes of amino acid synthesis. Past researches has shown that chitosan nanoparticles have the ability to inactivate and stabilize the enzymes that interact with them [18]. The results of previous research also showed that chitosan nanoparticles are effective in changing pathways and the result of amino acid synthesis process [19] [20].

In this experiment, the effect of chitosan nanoparticles on the types of amino acids produced in the rice kernel was not uniform. For instant, under the influence of one concentration like 1000 μL^{-1} of the chitosan nanoparticles spraying, some amino acids decreased (e.g., the Phe) and some increased (e.g., tyrosine), and others remain unchanged. The increasing or decreasing effect of chitosan nanoparticles on the amount of amino acids in the kernel depends on the reactivity of the amino acids precursors, the enzymes of the synthesis pathway, and the substrate level of the enzymes.

For example, some steps of the metabolic reaction are similar in the synthesis pathway of some amino acids such as phenylalanine and tyrosine, and only in the late steps of the synthesis pathway, the reactions are difference [21]. Because they should bind to a particular enzyme or a particular substrate, until to produce a different product. Thus, any factor that interfere with the synthesis steps and bind to the substrates or enzymes associated with the terminal steps of the synthesis pathway may play a role in

determining the type of amino acid and its rate of production, and even in the aberration and stopping of amino acid synthesis pathway [15]. Therefore, based on the results, weight loss of total amino acids produced in the rice kernel, especially the significant reduction of the Phe, could be due to changes in the enzymatic reactions which are related to the amino acid synthesis pathways.

The results showed that the amount of production of one type of amino acid varies at different concentrations of chitosan nanoparticles. Indeed, the effect of chitosan nanoparticles is related to changes in the nanoparticles concentration. It means that, the effect of varying the concentration of chitosan nanoparticles on the synthesis and production of an amino acid has different effects.

One of the most important causes of different effects (changes in the concentration of chitosan nanoparticles) on the production of an amino acid, is due to the effect of changing the total number of functional groups present in the molecular structure of the chitosan nanoparticles [22] [23]. Changing the total number of functional groups of the molecular structure of chitosan nanoparticles affects the reaction efficiency of chitosan nanoparticles, because they are functional groups of chitosan nanoparticles that interact with the amino acid synthesis pathway as effective and reactive agents [24]. Therefore, the participation rate of the functional groups is as an effective factor in guiding the reactions of the amino acid synthesis pathway and determining the product type of the synthesis process [25]. In other words, the number and types of reactive functional groups influence the choice and preference of the reaction of chitosan nanoparticles with a variety of regulator factors in the amino acid synthesis pathway.

In addition, other factors such as plant species, environmental conditions (temperature, soil moisture and pH), plant physiological growth stage, method of nanoparticle application, timing, and amount of chitosan nanoparticles can have different effects on the performance of chitosan nanoparticles [26] [27]. Therefore, the effects on plant growth and the metabolic response of the plant will not be uniform. Because amino acid synthesis reactions in plants are sensitive to regulators of amino acid biosynthetic pathways [28].

The other most important results in this study were the increased reaction of the PAL activity under the influence of the spraying application of chitosan nanoparticles in the rice kernel during grain growth and development stages. Although in all concentrations of chitosan nanoparticles, activity of the PAL was higher than the control treatment, the maximum activity of the PAL obtained with 1000 μL^{-1} of chitosan nanoparticles.

The increased activity of the PAL under chitosan has also been reported in other previous studies [29] and is in agreement with these results. Past researches also reported that by using spraying chitosan nanoparticles as an exogenous elicitor on plants, they enhanced the plant's defense system through stimulating the activity of defense and antioxidant enzymes and enhanced plant resistance to attack by pathogens [30]. In fact, plants affected by chitosan nanoparticles enhance the defense mechanism of the synthesis of antioxidant enzymes such as superoxide dismutase, catalases and peroxidases, as well as

the production of phenylalanine ammoniumase and other plant phytoalexins and secondary metabolites [31].

The results of a study on the reduction of the PAL activity suggest that compounds such as p-Coumarate, o-chlorosinamate, p-benzoate, 2-naphtholite and trans-cinnamate that are in place of the PAL can bind to the enzyme, then results in disruption and/or reduction of the phenylalanine ammoniasis activity [32]. For example, increasing the trans-cinamat level in the place of the PAL enzymatic can compete with the Phe for binding to the PAL, as a result inhibit the enzymatic activity of the PAL on the Phe. In fact, this inhibition does blockage metabolic pathway of the Phe for conversion to the other phenolic compounds [33]. However, this type of inhibition of the PAL effect on the Phe is not always inappropriate especially when plants are exposed to inappropriate changes in environmental conditions and biological and non-biological stresses to maintain plant life, the normal metabolic reactions and biosynthetic pathways of amino acids change temporarily, and these changes are the same plant responses to the effects of biological and abiotic stresses.

On the other hand, the activity of the PAL also depends on the amino acid concentration of phenylalanine, meaning that at lower concentrations than the Phe, it will have more enzymatic activity and this type of reaction is a result of the specificity of the the Phe accumulation, and as the feedback inhibitory cycle of the Phe is well known and it can affect the PAL activity [34].

Also, the results indicate that based on the quantities of essential amino acids produced in the rice kernel, the trend of changes in essential amino acids production was not uniformly under the spraying of chitosan nanoparticles and they had different reactions to each other. For these differences, it could be argued that the potential differences in the structure of molecules and compounds present at the site of metabolic reactions along with the functional groups of chitosan nanoparticles could be due to differences in amino acid production.

For example, according to the results of Table 4, amino acids that were raised to a concentration of $1000 \mu\text{L}^{-1}$ concentration of chitosan nanoparticles were structurally first of non-polar structures (e.g., alanine, isoleucine and valine), and later of polar structures and neutral (e.g., tyrosine and threonine). But, the amino acids that were reduced at $1000 \mu\text{L}^{-1}$ concentration of chitosan nanoparticles were more of the non-polar structures (e.g., phenylalanine, methionine, and tryptophan), followed by the negatively charged polar types (e.g., glycine and aspartic acid). Interestingly, the amino acid phenylalanine is a nonpolar and cyclic structure that is reduced by chitosan nanoparticles, and in contrast, tyrosine, it increases by a neutral and cyclic polar structure by chitosan nanoparticles. Therefore, the results show that in the first step, the polarity of molecule and, secondly, the electrical charge of the molecule determines the potent of reaction of compounds with chitosan nanoparticles at the site of metabolic reactions.

This kind of reaction between chitosan nanoparticles and other compounds, e.g., amino acid precursors, seems reasonable because, first, the molecular structure of chitosan nanoparticles affected by the

synthesis method, and second, the type and number of functional groups in the structure of chitosan nanoparticles, and third, the lack of an equal reaction of each of the functional groups due to the differences in the type and molecular structure of the substrate compounds in the amino acid biosynthesis pathway, which allows the reaction to be preferred and to decrease or increase the amino acid production. Therefore, the effect of chitosan nanoparticles spraying on the rice grain will result in a difference in amino acid production in the rice kernel.

Conclusion

Overall, the use of chitosan nanoparticles spraying during rice grain growth and development (spike onset, flowering onset and milky onset of grain) can affect the efficiency of metabolic reactions related to amino acid synthesis and amino acid production. Decrease of the Phe level and total protein, and likewise increase the PAL activity and tyrosine production in the rice kernel were the most important results of this study, and also they are necessary reasons for confirmation of metabolic reactions of the rice kernel due to the spraying application of chitosan nanoparticles. Also, rice kernel treated with $1000 \mu\text{L}^{-1}$ of chitosan nanoparticles produced minimum the Phe level and maximum of the PAL activity, and the concentration was identified as an optimum concentration in this experiment. The ability of chitosan nanoparticles to influence metabolic processes and amino acid synthesis depends on the type and the total number of functional groups in the chitosan nanoparticle structure. It also depends on the chemical properties, such as polarity and electrical charge of the molecules and compounds that interact with the nanoparticles in the amino acid synthesis pathway. Thus, the results of this study provide nearly the necessary documentation to support the hypothesis that the application of chitosan nanoparticles in the grain formation period is effective for reducing rice grain content of phenylalanine amino acid.

However, there is still insufficient documentation, and further studies are needed to determine the positive effects of chitosan nanoparticles on the control and effective reduction of the Phe in rice and other cereals, for example, there still are questions:

1. By changing environmental conditions or changing plant species, can the reduction of the Phe level under the application of chitosan nanoparticles be repeated?
2. How can chitosan nanoparticles be used to prevent reduced total protein production in the grains?
3. How will the change in the timing and frequency of chitosan nanoparticle spraying affect the amino acid synthesis processes?
4. Do nanoparticles made of chitosan derivatives can enhance the efficiency of nanoparticles in controlling amino acid synthesis processes?

Materials And Methods

chitosan nanoparticles synthesis

Chitosan nanoparticles were synthesized according to the modified method of Kashyap *et al.* (2015) [18]. 0.2 g of chitosan with 85% deacetylation was dissolved in 27 ml of 1% acetic acid for 2–3 h at 50 °C. Then, 9 ml of the tripolyphosphate solution (which was prepared from dissolving 21 mg of tripolyphosphate in 30 ml distilled water), was added dropwise to the chitosan solution under a magnetic stirrer. The pH was adjusted to 6.5–7 with sodium hydroxide solution [35].

Method of Planting rice

The methods of planting rice are very different even in one country. The method used for planting rice in this research was a locally method. The rice seeds (*Oryza sativa* L., cultivar Sang-Jo) were obtained from Rasht Biotechnology Institute. The rice seed was soaked in water for 3 days, then poured into a plastic bag and surrounded with a cloth, and some water was poured on the seeds daily, germinating after 4 days. They were then planted in beds (a special farm that is a very small and very fertile farm that is very suitable for seedling growth). After 35 days, the seedlings were transplanted by hand to the plots (40×40 cm) in the main field (in the fields of Siahkal city, Gilan province, Iran).

Preparation of treatments and harvest time

After synthesis of the chitosan nanoparticles, the treatments were prepared in different concentrations of chitosan nanoparticles (0 as control, 500, 1000, 1500 $\mu\text{g L}^{-1}$) as experimental treatments, and the concentrations were dissolved in tap water, and later in the physiological stages of grain growth (onset spike, onset flowering, onset milky of grain) during sunset on the aerial parts (especially the spikes) of the rice plant were sprayed. After complete ripening of rice (about 4 to 5 months), the spikes were harvested and the experimental variables (amino acids production, the PAL activity and total protein of grain) were measured in the rice kernel.

Determination of total protein

Total protein in the rice kernel was measured by standard Bradford assay [36]. Bovine serum albumin was used as a standard to test protein concentration of each extract.

Determination of amino acids

Amino acids in the rice kernel were determined according to the modified Qing-qing Yang *et al.* (2015) [37]. From each experimental treatment, 100 mg of powdered rice kernel was measured, and later, 1 ml of 80% ethanol was added to each sample, and kept at 4 °C. The samples were placed with a thermomixer at 80 °C for one hour at 700 rpm, and they were then allowed to be near ambient temperature. The samples were then centrifuged at 14,000 rpm for 5 minutes at 4 °C. The supernatant was poured into another tube, the tubes were completely open and covered with paraffin and placed in a freeze-dryer overnight. After complete lyophilization, the samples were stored at -20 °C. To the tubes containing the sample, one ml of distilled water was added and filtered with 0.22 μm syringe filters. Then 250 μL of the samples were removed and poured into another tube and then 200 μL of borate buffer and 100 μL of ortho

phthalate dihydrate (OPD) were added to these tubes. The samples were then vortexed for 120 seconds. Later, 50 µl of HCL 0.5 mM, was added to each samples and dissolved with vortex for 15 seconds, and then by syringe was added to the HPLC instrument and the column detail was HALO 5 µm, C18.

Determination of the Phenylalanine ammonia-lyase (PAL)

The PAL activity was determined according to the Aydas et al. (2013) [21]. 0.3 g of rice kernel was pulverized in 6.5 ml of buffer (Tris-hydrochloric acid buffer 50 mM (pH= 8.8) which was containing 15 mM of beta mercaptoethanol) and the mixture was then centrifuged at 5000 rpm for 30 min. Supernatant was used to measure the activity of the PAL and total protein. One ml of extraction buffer, 500 µl of 10 mM phenylalanine, 400 µl of water, 100 µl of plant extract were mixed and placed in a warm bath at 37 °C for one hour. Then 500 µl hydrochloric acid, and also, 15 ml of ethyl acetate solution were added to the final mixture. Then an oily phase, which is the kinamic acid, was dissolved in 3 ml of 0.05 M NaOH, and the cinnamic acid concentration was obtained by measuring the absorbance by spectrophotometer at 290 nm using an extinction coefficient of 9500 molar cm⁻¹. The activity of the PAL was determined by the rate of conversion of the Phe to trans-cinnamic acid. One unit of the PAL activity is equivalent to one micromole of cinnamic acid produced in one minute [38].

Experimental data were first normalized by the Jarkio test; the data were then analyzed statistically by using SPSS software version 20. Comparisons of treatments averages were performed with Duncan's test at 99% and 95% probability levels, and also the data were plotted using Excel.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The data set generated and analyzed during the present study is not available to the public due to data related to a project supported by Shahid Beheshti University, and the authors signed a confidential letter to protect the data set. In fact, this manuscript offers a new platform for changing the synthesis pathways of amino acids.

Competing interests

The authors have declared that there is no conflict of interest.

Funding

The Shahid Beheshti University has supported this manuscript in the form of a project to financially support student dissertations and provide laboratory and greenhouse facilities.

Authors' contributions

For preparation of the manuscript, all authors have an equal share in the analysis, interpretation of results, and all authors have read and approved the manuscript.

Acknowledgment

This research was carried out in analysis biomaterials center of plant sciences department, Shahid Beheshti University that is sincerely acknowledged.

References

1. Cleary, M.A. and R. Skeath, *Phenylketonuria*. Paediatrics and Child Health, 2019.
2. *Leaf-nosed bat*, in *Encyclopædia Britannica*. 2009, Encyclopædia Britannica Online.
3. Winn, S.R., et al., *Blood phenylalanine reduction corrects CNS dopamine and serotonin deficiencies and partially improves behavioral performance in adult phenylketonuric mice*. Molecular genetics and metabolism, 2018. **123**(1): p. 6-20.
4. Soltanizadeh, N. and L. Mirmoghtadaie, *Strategies Used in Production of Phenylalanine-Free Foods for PKU Management*. Comprehensive Reviews in Food Science and Food Safety, 2014. **13**(3): p. 287-299.
5. Yang, P., et al., *Proteome analysis of rice uppermost internodes at the milky stage*. Proteomics, 2006. **6**(11): p. 3330-3338.
6. Mohsen, S.M., et al., *Quality characteristics improvement of low-phenylalanine toast bread*. International journal of food science & technology, 2010. **45**(10): p. 2042-2051.
7. Tien Lea, D., H. Duc Chua, and N. Quynh Lea, *Improving nutritional quality of plant proteins through genetic engineering*. Current genomics, 2016. **17**(3): p. 220-229.
8. Wang, G., et al., *Fortifying horticultural crops with essential amino acids: a review*. International journal of molecular sciences, 2017. **18**(6): p. 1306.
9. Huang, M., et al., *Amino acid content in rice grains is affected by high temperature during the early grain-filling period*. Scientific reports, 2019. **9**(1): p. 2700.
10. Zeier, J., *New insights into the regulation of plant immunity by amino acid metabolic pathways*. Plant, Cell & Environment, 2013. **36**(12): p. 2085-2103.
11. Hildebrandt, T.M., et al., *Amino acid catabolism in plants*. Molecular plant, 2015. **8**(11): p. 1563-1579.
12. Zhang, X. and C.-J. Liu, *Multifaceted regulations of gateway enzyme phenylalanine ammonia-lyase in the biosynthesis of phenylpropanoids*. Molecular Plant, 2015. **8**(1): p. 17-27.

13. Prasad, R., A. Bhattacharyya, and Q.D. Nguyen, *Nanotechnology in sustainable agriculture: recent developments, challenges, and perspectives*. Frontiers in microbiology, 2017. **8**: p. 1014.
14. Elsoud, M.M.A. and E. El Kady, *Current trends in fungal biosynthesis of chitin and chitosan*. Bulletin of the National Research Centre, 2019. **43**(1): p. 1-12.
15. Hidangmayum, A., et al., *Application of chitosan on plant responses with special reference to abiotic stress*. Physiology and molecular biology of plants, 2019. **25**(2): p. 313-326.
16. Sreekumar, S., et al., *Parameters influencing the size of chitosan-TPP nano-and microparticles*. Scientific reports, 2018. **8**(1): p. 4695.
17. Islam, N., I. Dmour, and M.O. Taha, *Degradability of chitosan micro/nanoparticles for pulmonary drug delivery*. Heliyon, 2019. **5**(5): p. e01684.
18. Wang, K., *Enzyme immobilization on chitosan-based supports*. Chitosan-Based Hydrogels, 2011: p. 339-406.
19. Chandra, S., et al., *Chitosan nanoparticles: a positive modulator of innate immune responses in plants*. Scientific reports, 2015. **5**: p. 15195.
20. Younes, I. and M. Rinaudo, *Chitin and chitosan preparation from marine sources. Structure, properties and applications*. Marine drugs, 2015. **13**(3): p. 1133-1174.
21. Parthasarathy, A., et al., *A three-ring circus: metabolism of the three proteogenic aromatic amino acids and their role in the health of plants and animals*. Frontiers in molecular biosciences, 2018. **5**: p. 29.
22. Aranaz, I., et al., *Functional characterization of chitin and chitosan*. Current chemical biology, 2009. **3**(2): p. 203-230.
23. Aljawish, A., et al., *Enzymatic synthesis of chitosan derivatives and their potential applications*. Journal of Molecular Catalysis B: Enzymatic, 2015. **112**: p. 25-39.
24. Rajasree, R. and K. Rahate, *An overview on various modifications of chitosan and it's applications*. Int J Pharm Sci Res, 2013. **4**(11): p. 4175.
25. Hadwiger, L.A., *Multiple effects of chitosan on plant systems: solid science or hype*. Plant science, 2013. **208**: p. 42-49.
26. Malerba, M. and R. Cerana, *Recent advances of chitosan applications in plants*. Polymers, 2018. **10**(2): p. 118.
27. Brasili, E., et al., *A non-targeted metabolomics approach to evaluate the effects of biomass growth and chitosan elicitation on primary and secondary metabolism of Hypericum perforatum in vitro roots*. Metabolomics, 2014. **10**(6): p. 1186-1196.
28. Pratelli, R. and G. Pilot, *Regulation of amino acid metabolic enzymes and transporters in plants*. Journal of Experimental Botany, 2014. **65**(19): p. 5535-5556.
29. Rabêlo, V.M., et al., *The foliar application of a mixture of semisynthetic chitosan derivatives induces tolerance to water deficit in maize, improving the antioxidant system and increasing photosynthesis and grain yield*. Scientific reports, 2019. **9**(1): p. 8164.

30. Malerba, M. and R. Cerana, *Chitosan effects on plant systems*. International Journal of Molecular Sciences, 2016. **17**(7): p. 996.
31. Siddaiah, C.N., et al., *Chitosan nanoparticles having higher degree of acetylation induce resistance against pearl millet downy mildew through nitric oxide generation*. Scientific reports, 2018. **8**(1): p. 2485.
32. Ritter, H. and G.E. Schulz, *Structural basis for the entrance into the phenylpropanoid metabolism catalyzed by phenylalanine ammonia-lyase*. The Plant Cell, 2004. **16**(12): p. 3426-3436.
33. Kurepa, J., et al., *Modulation of auxin and cytokinin responses by early steps of the phenylpropanoid pathway*. BMC plant biology, 2018. **18**(1): p. 278.
34. Kong, J.-Q., *Phenylalanine ammonia-lyase, a key component used for phenylpropanoids production by metabolic engineering*. RSC Advances, 2015. **5**(77): p. 62587-62603.
35. Kashyap, P.L., X. Xiang, and P. Heiden, *Chitosan nanoparticle based delivery systems for sustainable agriculture*. International journal of biological macromolecules, 2015. **77**: p. 36-51.
36. Kruger, N.J., *The Bradford method for protein quantitation*, in *The protein protocols handbook*. 2009, Springer. p. 17-24.
37. Yang, Q.-q., et al., *Biofortification of rice with the essential amino acid lysine: molecular characterization, nutritional evaluation, and field performance*. Journal of experimental botany, 2016. **67**(14): p. 4285-4296.
38. Aydaş, S.B., S. Ozturk, and B. Aslım, *Phenylalanine ammonia lyase (PAL) enzyme activity and antioxidant properties of some cyanobacteria isolates*. Food chemistry, 2013. **136**(1): p. 164-169.

Figures

Figure 1

Average of the Phe (A); average of the PAL activity (B); average of the total protein (C) level in the rice kernel by different concentrations of chitosan nanoparticle spraying. Capital and lowercase letters denote a significant difference at $p < 0.01$, $p < 0.05$, respectively. Error bars denote standard error.