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Effect of natural PAL-enzyme on the quality of egg white and mushroom flour and study its impact on the expression of PKU related genes and phenylalanine reduction in mice fed on

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ABSTRACT

PKU patients react to therapy with a low phenylalanine diet, but adherence to this diet is troublesome, subsequently the expansion of alternative ways is demand. Phenylalanine ammonia lyase (PAL) is one of this ways, which converts phenylalanine to harmless metabolites; trans-cinnamic acid and ammonia. In the current study, the extraction of PAL enzyme was used to investigate the efficiency for production of functional PKU egg white and mushroom flour with good quality by evaluation of colour characteristics, determination of phenylalanine concentrations and genetic materials expression of PKU related genes and DNA damage. Results indicated that the PAL enzyme treated of egg white and mushroom flour was stable colour and the calculated reduction per cent in phenylalanine concentration from female mice fed on untreated and PAL–treated samples was 22.77% in egg white and 31.37% in mushroom flour. Also, the results revealed that female mice fed on diet contained treated egg white exhibited low expression levels of PKU exons (3, 6, 7, 11, and 12) and low DNA damage which were similar to those in control mice.
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1. Introduction

Phenylketonuria (PKU; OMIM 261600), the "poster child" for much of our understanding of metabolic disorders, affords a good model for effective management through dietary handling. PKU is an autosomal recessive genetic disorder, resulting from a defect in phenylalanine hydroxylase (PAH) or secondarily due to impairment in tetrahydrobiopterin metabolism, which is a cofactor for PAH [7]. All PKU patients are characterized by increasing and growing of phenylalanine (phe.) and its metabolites in blood and urine also, characterized by a severe mental retardation with reduced growth, microcephaly, neurological signs and behavioural problems also reduced hair skin, and iris pigmentations are observed [40]. Moreover, stress resulting from oxidation is coinciding with physiology and pathology of PKU which induce a neurodegeneration in this disease [37,29,31]. This neurodegeneration induces oxidative break in several kinds of cells such as pro-

tein, RNA, DNA and lipids [14]. In this regard, when Schulpis et al. [34] analysed the serum of PKU patients found correlation of Phe levels and DNA adducts (8-OHdG) which produced from exposure to oxidative stress. Furthermore, hyperphenylalaninemia was found in the DNA molecules extracted from animal models of PKU [36]. Dietary therapy with restriction of protein remains the cornerstone for treatment of PKU patients in order to keep blood phenylalanine level within safe limit that means no eggs, no cheese and no meat. Their food is depended on a special formula they eat at meal times, supplemented by fruits and vegetables. The diet therapy should be beginning early after birth; therefore, newborn screening (NBS) programmes are necessary to identify the PKU patients early [40].

Adherence to diet therapy is difficult especially in adolescence due to the off odour and bitterish taste of the amino acid recipes in addition to strong rival pressure to eat normal foods; therefore, new dietary routes are necessary to recover the palatability and variety of the low-Phe diet to promote compliance, metabolic monitoring and quality of life for PKU patient [20,42] such as, large neutral amino acids supplementation [23], a medical foods based upon glycomacropeptide, a naturally low-Phe protein, [25]

pharmacologic chaperones, cofactor. 5,6,7,8-tetrahydrobiopterin (BH4). Lastly, the subcutaneous injection of phenylalanine ammonia lyase (PAL) to convert blood phenylalanine to harmless metabolites, trans-cinnamic acid and ammonia [4,19]. Two phase's trials I and II have revealed the injected recombinant PAL which produced in *E. coli* and conjugated with PEGylation can reduce phe. level [12,13].

From literature effect of different traditional and nontraditional processing methods on foods to reduce phenylalanine content was rarely found. Also, there is no effective method can be suggested for supplying phenylalanine hydroxylase to phenylketonuria patients. The treatment for the food now in use can be producing a free amino acid mixture from which phenylalanine has been unnaturally removed. It is pointed out that the amino acid mixture is commonly artificial as food for human [2].

Although the frequently question is to produce naturally occurring proteins containing a very low quantity of phenylalanine, it could be an effort to make up such a substance by making use of new techniques. Yamashita et al. [43] explain how to apply a series of enzymatic processes including the so-called plastein reaction for prepare two kinds of peptide-type with low-phenylalanine substances. Not in animals or bacteria, but in some fungi and in all higher plants analyzed to date has been contains PAL enzyme activity. One of the best studied is PAL enzyme in higher plants because of the important of PAL enzyme in the formation of lignin, isoflavonoids, and other secondary metabolites [8]. PAL activity has been extracted in many species of plants, such as monocots, dicots, gymnosperms [24]. Moreover, PAL cDNAs were isolated, and sequenced from different banana cultivars by Alvarez et al. [3].

Egg white and edible mushrooms are considered protein food products [5,30]. The shelf life of oyster mushroom processed is restricted to a few days, related to the enzymatic browning during storage. It has been supposed that the PAL increases during the minimum processing due to the damages caused in the cells [32]. Many modern processes for food production employ enzymes as important processing aids to obtain good food quality (especially colour characteristics) [10,38].

Too little of research papers were carried out in the field of PAL enzyme extract to reduce of phenylalanine concentration in food processing. In the present paper, we study the characterization and quality of crude Phenylalanine Ammonia-lyase enzyme activity (PAL) from banana fruit (*Musa cavendishii* L., cv. Enana) to produce a functional PKU egg white and mushroom flour with good quality by evaluation of colour characteristics, determination of phenylalanine concentrations, genetic materials expression of PKU related genes and DNA damage.

2. Material and methods

2.1. Fruit samples

Banana (*Musa cavendishii*, cv. Enana) fruits grown in Egypt were obtained from a local supermarket in Cairo. Banana fruits were placed in refrigerator at 3–4 °C for 4 h until used.

2.2. Enzyme preparation

Using blender, 10 g banana fruits were homogenized in 100 ml of cold acetone and the soluble residue filtered and dried under vacuum. It was extracted at 4 °C by gentle stirring with 50 ml of extraction buffer which contained 100 mM sodium borate buffer (pH 8.8), 2 mM EDTA (ethylendiamintetraacetic acid) and PVPP (polyvinylpolypyrrolidone) at 100 g kg^{-1} of the fruit fresh weight. After 1 h of extraction, the solution was filtered through two layers of cheese clothes and centrifuged at 5000 rpm at 4 °C for 15 min [41].

2.3. PAL enzyme activities assay

PAL enzyme activities in the buffer supernatant were determined by the production of trans-cinnamic and p-coumaric acids, respectively, from 1-phenylalanine (PAL) during 1 h at 30 °C, as measured by the absorbance change at 290 nm [41]. The assay mixture contained 1.5 ml 30 mM sodium borate buffer (pH 8.8) and 0.5 ml buffer supernatant, and 1 ml 0.015 M L-phenylalanine for PAL assays. The substrates (L-phenylalanine for PAL) were added after 10 min of pre incubation and the reactions stopped with 0.1 ml 6 M HCl. Assays were performed in triplicate. L-phenylalanine was obtained from Sigma (St. Louis, MO, USA)

2.4. Food processing

2.4.1. Egg white processing
Red chicken egg was obtained from a commercial super market, Cairo, Egypt. Freshly egg white separated from the egg yolk. However, egg white was treated by 1% PAL enzyme and incubated at room temperature for 2 h then added 0.05% SO$_2$ to stop of the activity of PAL enzyme.

2.4.2. Mushroom flour production
Fresh mushroom (*Pleurotus ostreatus*) was obtained from a commercial mushroom farm at Cairo, Egypt. The fresh mushroom obtained was pre-treated with PAL enzyme (1%) and incubated at room temperature for 2 h then added 0.05% SO$_2$ to stop the activity of PAL enzyme and dried in an oven dried (60 °C) using hot air oven (SheL, LAb 1370 FX, Shel Don manufacturing, Inc. and Germany) for 8hr. to obtain a completely dried sample. Dehydration was continued until the moisture content of mushroom slices reached about 14%, using modified method of Parab et al. [28], it was then pulverized, sieved and stored in polyethylene bag at room temperature at 20 °C for use afterwards.

2.5. Nutrition experiment

Fifty Swiss albino female mice were obtained from the animal house of the National Research Centre, Dokki, Giza, Egypt and immediately after weaning, they were classified into 5 groups of 10 mice each as follows:

Group 1: used as a control group with normal feeding (22% protein, 3.48% fat and 3.71% dietary fibre as 60% yellow corn, 34% soya bean, calcium phosphate, sodium chloride, calcium chloride, oil soya, vitamins, antitoxins and molasses). Group 2: fed on raw egg white without any treatment. Group 3: fed on PAL enzyme treated egg white. Group 4: fed on dried mushroom flour without any treatment. Group 5: fed on dried PAL enzyme treated mushroom flour.

All groups were fed with 0.5 mg/kg m body weight for one month. Animals were housed under temperature- and light-controlled conditions with standard laboratory rodent chow and water provided ad libitum. Animal procedures were performed in accordance with the Ethics Committee of the National Research Centre and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (publication no. 85-23, revised 1985). Diet and water were provided ad libitum.

2.6. Determination of amino acids in egg white and mushroom flour

All amino acids were analysis for untreated and PAL enzyme treated egg white and mushroom flour samples according to the method of A.O.A.C. [1].

% reduction = [(phenylalanine content in PAL-enzyme treated
/phenylalanine content in untreated sample)] ∗ 100

2.7. Colour characteristics determination of egg white and mushroom flour

Colour of untreated and PAL-enzyme treated egg white and mushroom flour was measured using spectro colourimeter (Tristimulus Colour Machine) with the CIE lab colour scale (International Commission on Illumination) as mentioned by Hunter [16] and Sapers and Douglas, [33]. Colour of Fresh egg white and mushroom flour samples was measured using a HunterLab colourimeter Hunter a^*, b^* and L^*. Parameters were measured with a colour difference meter using a spectro-colourimeter (Tristimulus Colour Machine) with the CIE lab colour scale (Hunter, Lab Scan XE - Reston VA, USA) in the reflection mode. The instrument was standardized each time with white tile of Hunter Lab Colour Standard (LX No. 16379): X = 72.26, Y = 81.94 and Z = 88.14 (L^* = 92.46; a^* = −0.86; b^* = −0.16). The instrument (65°/0° geometry, D25 optical sensor, 10° observer) was calibrated using white and black reference tiles. The colour values were expressed as L^* (lightness or brightness/darkness), a^* (redness/greenness) and b^* (yellowness/blueness). The Hue $(H)^*$, Chroma $(C)^*$ and Browning Index (BI) were calculated according to the method of Palou et al. [27] as follows:

$$H^* = \tan{-1}[b^*/a^*] \tag{1}$$

$$C^* = \text{square root of } [a2^* + b2^*] \tag{2}$$

$$BI = [100(x - 0.31)]\ 10.72 \tag{3}$$

where $X = (a^* + 1.75\ L^*)/(5.645\ L^* + a^* - 3.012b^*)$

$$\Delta E = (\Delta a^2 + \Delta b^2 + \Delta L^2)^{1/2} \tag{4}$$

where all values were recorded as the mean of triplicate readings.

2.8. Non-enzymatic browning determination

Non-enzymatic browning was measured spectrophotometrically by 4054 - UV/Visible spectrophotometer, (LKB-Biochrom Comp., London, England), as absorbance at 420 nm using ethanol as a blank according to the method of Stamp and Labuza [39] and Birk et al. [6].

2.9. Determination of phenylalanine concentration in dried blood spot using Tandem mass spectrometry

Chemicals included MassChrom Reagent Kit for the LC-MS/MS analysis of amino acids and acylcarnitines from dried blood spot-\non derivatized, which contained; mobile phase, lyophilized internal standard, rinsing solution, extraction buffer, 96 well plates V bottomed, protective sheets, pierceable adhesive seal and amino acids, acylcarnitine dried blood spot control bi-level(I + II)-Gmbh, Guthrie filter paper cards (S&S 903; Schleicher and Schull, Dassel, Germany) were used to collect blood samples. The blood samples from female mice were spotted on Guthrie filter paper cards, left to dry at room temperature for at least 24 h. The procedures of extraction and mass analysis were applied according to instruction of manufacture chromsystems; briefly, 3 mm dried blood spot disk was punched out of the filter card from level I (target value of

phenylalanine 167 μmol/l, range 107–227) level II (target value of phenylalanine 564 μmol/l, range 348–780) and sample into a well of a microtiter plate. For the extraction: 100ul of the reconstituted internal standard with known concentration of phenylalanine-D5 (6.52 μmol/l) was added for each well. Sealed the microtiter plate and agitated at 600 rpm for 20 min. at ambient temperature. The supernatant was transferred into a new v-bottomed well plate. Sealed the plate with a piercable adhesive seal, 10ul of the eluate was injected into the LC-MS/MS system, the concentration of the analyte in the sample was calculated according to: The concentration C Analyte, (μmol/l) = (signal intensity of analyte A in the sample spectrum/single intensity of the internal standard IS in the spectrum of sample) X sample related concentration C of the internal standard.

2.10. Mass spectrometry (MS)

A waters Xevo triple quadrupole mass spectrometer (Milford, USA) equipped with electrospray ionization (ESI) source in positive ion mode was used for sample analysis, a multiple reaction monitoring (MRM) was used for analytes and internal standards (phenylalanine transition 165.98/120 and phenylalanine-D5 171.12/125.2). The automatic sampler and the pump of an LC apparatus were used to inject sample and mobile phase to MS, but no LC column was needed. The MassLynx V4.1 and NeoLynx were used for data acquisition and processing of MS analysis. A designed MS/MS program for automatic profiling of acylcarnitines and amino acids was used.

2.11. Gene expression analysis

2.11.1. RNA extraction

The total RNA from brain tissues of female mice was extracted using TRIzol® Reagent (Invitrogen, Germany) according to the manufacturer's instructions with minor modifications. Aliquots were used immediately for reverse transcription (RT), otherwise they were stored at −80 °C [11].

2.11.2. Reverse transcription reaction

Complete Poly(A)+ RNA isolated from brain tissues was reverse transcribed (RT) into cDNA in a total volume of 20 μl using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Germany). The reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for cDNA amplification through quantitative Real-time polymerase chain reaction (qPCR) [17,18].

2.11.3. Real-time PCR

One step's real-time PCR cycler (Applied Biosystem, USA) was used to determine the brain cDNA copy number. PCRs were set up in 25 μl reaction mixtures containing 12.5 μl 1× SYBR® Premix Ex Taq™ (TaKaRa, Biotech. Co. Ltd.), 0.5 μl 0.2 μM sense primer, 0.5 μl 0.2 μM antisense primer, 6.5 μl distilled water, and 5 μl of cDNA template. Each experiment included a distilled water control. The sequences of specific primer of the genes used are listed in Table 1. At the end of each qPCR a melting curve analysis was performed at 95.0 °C to check the quality of the used primers. The relative quantification of the target (PKU exons 3, 6, 7, 11 and 12) to the reference (β-actin) was determined using the $2-\Delta\Delta CT$ method.

2.11.4. DNA fragmentation analysis

Apoptotic DNA fragmentation in liver tissues of female mice was qualitatively analysed by detecting the laddering pattern of nuclear DNA as described according to Lu et al. [21].

Table 1
Effect of PAL-enzyme on colour characteristics, parameters and non-enzymatic browning (A420 nm) in egg white and mushroom flour.

Treatments	L^*	a^*	b^*	ΔE	A_{400nm}	A_{420nm}	C^*	H^*	BI
Egg white	13.61	−1.55	1.28	78.85	1.86	1.56	2.01	39.56	1.91
STDEV	0.08	0.04	0.04	0.21	0.04	0.01	0.05	0.14	0.14
Mush flour	73.02	6.05	27.16	74.22	15.82	18.92	27.83	77.45	94.12
STDEV	0.15	0.04	0.09	0.06	0.1	0.08	0.08	0.08	0.21
PAL-egg white	15.92	−2.5	2.63	76.3	1.96	2.05	3.62	47.01	10.34
STDEV-EW	0.10	0.15	0.07	1.05	0.03	0.04	0.15	1.03	0.49
PAL-Mush flour	73.05	6.34	27.43	77.37	19.23	22.64	28.16	77.00	95.67
STDEV	0.18	0.066	0.15	0.15	0.065	0.05	0.16	0.06	0.91

±Standard Deviation (SD)/SQR^2 (n), where, n = 3.

3. Results and discussions

3.1. Phenylalanine ammonia layase (PAL) enzyme activity in fresh egg white and mushroom slices

Fig. 1, showed that fresh egg white and mushroom flour had different activities of enzymes such as phenylalanine ammonia layase (PAL) being 0.41 "Unit/g/h" in fresh mushroom sample and 0.0062 "Unit/g/h" in fresh egg white sample. PAL enzymes are responsible for various changes in chemical ingredients that will affect the quality characteristics and deterioration of fresh or processed mushroom. However, enzyme activity of phenylalanine ammonia layase (PAL) in mushroom showed greater activity than in egg white (Fig. 1). As seen in Fig. 1, PAL enzyme activity increased noticeable in mushroom samples.

3.2. Effect of PAL enzyme treatment on phenylalanine concentration "Unit/g/h" in egg white and mushroom flour using amino acid analyser

Fig. 2 represents the content of phenylalanine concentration in the studied untreated and PAL – treated egg white and mushroom flour samples. The phenylalanine content was 0.8 and 0.14 "Unit/g/h" in fresh egg white and mushroom flour samples. But, the phenylalanine content was decreased as a result of PAL-enzyme activity to 0.71 and 0.09 in treated egg white and mushroom flour samples. The percentage reduction in phenylalanine concentration was 11.25% and 35.71% in Pal-enzyme treated egg white and mushroom flour, respectively.

3.3. Effect of PAL-enzyme on colour characteristics, parameters and non-enzymatic browning (A420 nm) in egg white and mushroom flour

Using the Hunter Lab colour scale to measure the surface colour of untreated and PAL-enzyme treated egg white and mushroom flour. The Hunter colour values of untreated and PAL-enzyme treated egg white and mushroom flour were determined immediately after processed. The results of the Hunter Lab colours showed that the changes in a^* values were inversely relationship to the changes in L^* values. Absorption at 420 nm, the L^*, a^*, b^* values colour parameters, hue angle (H^*), chroma (C^*) and BI values were found to be appropriate indicators for the brown pigment composition because of nonenzymatic browning after processing, as seen in Table 1. The a^* values and BI for fresh egg white were low (−1.55 and 1.91) in contrast to high values for untreated mushroom flour (6.05 and 94.12) sample. Whereas, the a^* values and BI for PAL enzyme egg white were low (−2.5 and 10.34) in contrast to high values for PAL enzyme mushroom flour (6.34 and 95.67) sample under all tested conditions, fresh egg white showed much lower efficient values based on L^*, a^*,b^*, BI, C^*, H^* and A420 nm than ΔE values measurements, whereas the mushroom flour sample behaved an opposite trend. Such a trend is in agreement with previous studies of Ozoglu and Bayindirh [26]. These results explained that the increased browning was related to PPO enzyme activity in mushroom flour samples than in fresh egg white samples, as seen in Table 1. According to our results, the main colour changes by increasing of the BI and a^* value in mushroom flour, which were in high correlation to browning measurement. Other colour parameters, such as hue angle and chroma, also explained that egg white and mushroom flour caused a slight colour changes.
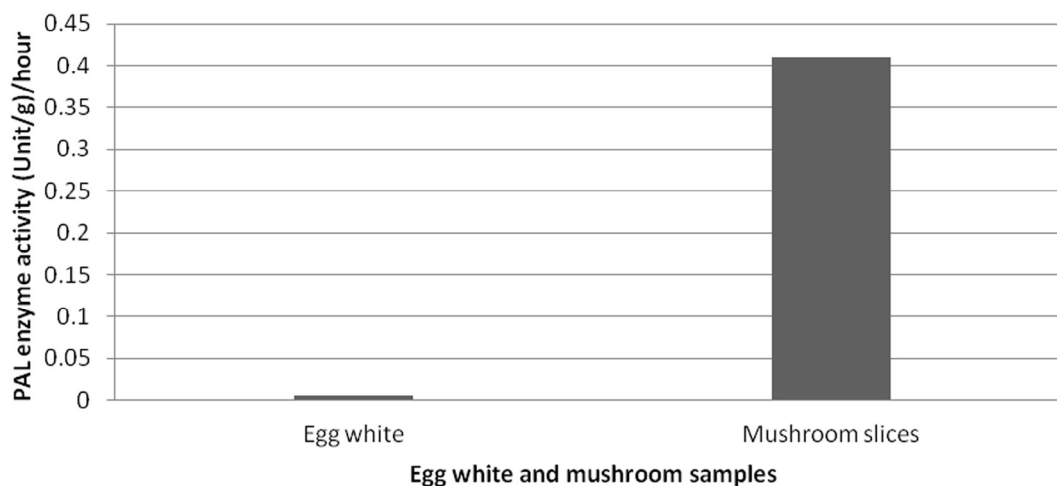

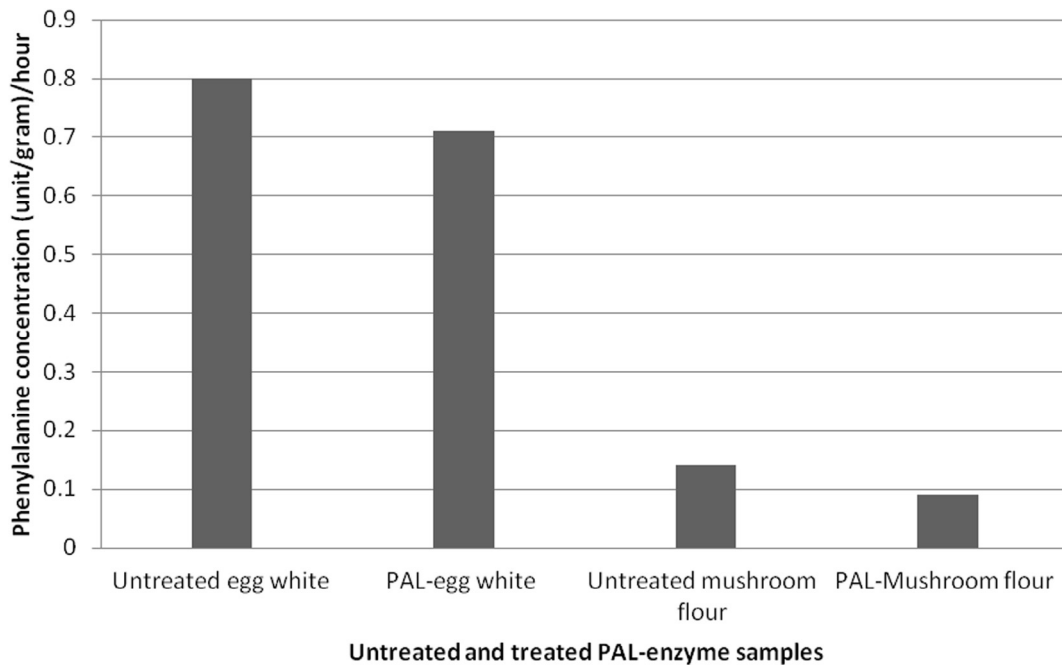**Fig. 1.** Phenylalanine ammonia layase activity (Unit/g)/h in fresh egg white and mushroom slices.

Fig. 2. Effect of PAL-enzyme on phenylalanine concentration in egg white and mushroom flour.

Our results are consistent with those of Palou et al. [27] and Ozoglu and Bayindirh [26]. In general, it was showed that mushroom flour samples had the higher increased in colour as optical density (A420 nm) of nonenzymatic browning compared with fresh egg white samples.

Hunter colour (L*, a* and b*) values were increased, while R420 (reflectance) were decreased in PAL treated egg white and mushroom flour samples compared with untreated samples. This is perhaps due to release of carotenoids and anthocyanins as a result of PAL enzyme addition. While, the addition of PAL enzyme resulted in an increase in the L* and b*-values but decrease in a*-value and R420 nm.

The increasing in colour browning values (as A420 nm) could be referred to the reaction occurred between amino groups and active carbonyl groups (Maillard reaction) after oven drying process. From our results, it could be concluded that pretreated oven drying process did not affect non enzymatic browning with optical density (A420 nm). However, the values of non enzymatic browning (A420 nm) confirm the data obtained for oven drying process showed higher values (18.92) in mushroom flour compared with (2.015) fresh egg white. Results explained the linear and quadratic effects of the independent variables for the colour characteristics L*, a*, b*, ΔE, Chroma, BI and Hue angle, are shown in Table 1.

3.4. Effect of PAL enzyme treated egg white and mushroom flour on phenylalanine concentration in blood spot mice by Tandem mass

Among inherited amino acid metabolic disorders, PKU is the most prevalence one. It estimated that overall prevalence of PKU in Arabic population is up to 1:6000 [35]. The therapy is based on lifelong protein restricted diets which it associated with many shortcomings like a deficiency in several nutrients that are necessary for brain development. Beside most phenylalanine formulas are organoleptic properties sufficiently unsatisfactory that compliance with the treatment is compromised [22]. The present study is an attempt to find potential therapeutic pathway for PKU using dietary regimens for allocating phenylalanine in order to overcome the shortages in the diet therapy.

Normal mice were used as control due to the absence of human mouse model for phenylalanine hydroxylase (PAH) deficiency (pahenu1/enu2) in my country Egypt.

The result showed that, the average of phenylalanine concentration (μmol/l) in dried blood spot, using tandem mass spectrometry with non-derivatized method, for control group was 70.32 μmol/l (with standard deviation ±11.9 and standard error ±3.77). While in case of untreated egg white group, the concentration of phenylalanine was 84.46 μmol/l (with standard deviation ±14.13, standard error ±4.47) and decreased to 65.23 μmol/l after treatment with PAL enzyme (with standard deviation ±10.86 and standard error ±3.44,). Wherever, the average concentration of phenylalanine in mushroom flour group was 79.16 μmol/l (with standard deviation ±5.15 and standard error ±1.63) which depleted to 54.33 after treatment with PAL enzyme (with standard deviation ±14.13 and standard error ±4.47) as shown in Table 2.

Also showed, the per cent of calculated reduction in phenylalanine concentration between untreated group and PAL enzyme – treated group was 22.77% in egg white and 31.37% in mushroom flour, as seen in Fig. 3.

Table 2
Phenylalanine concentration (μmol/l) in blood mice fed on untreated and PAL treated egg white and mushroom flour using Tandem Mass spectrometry.

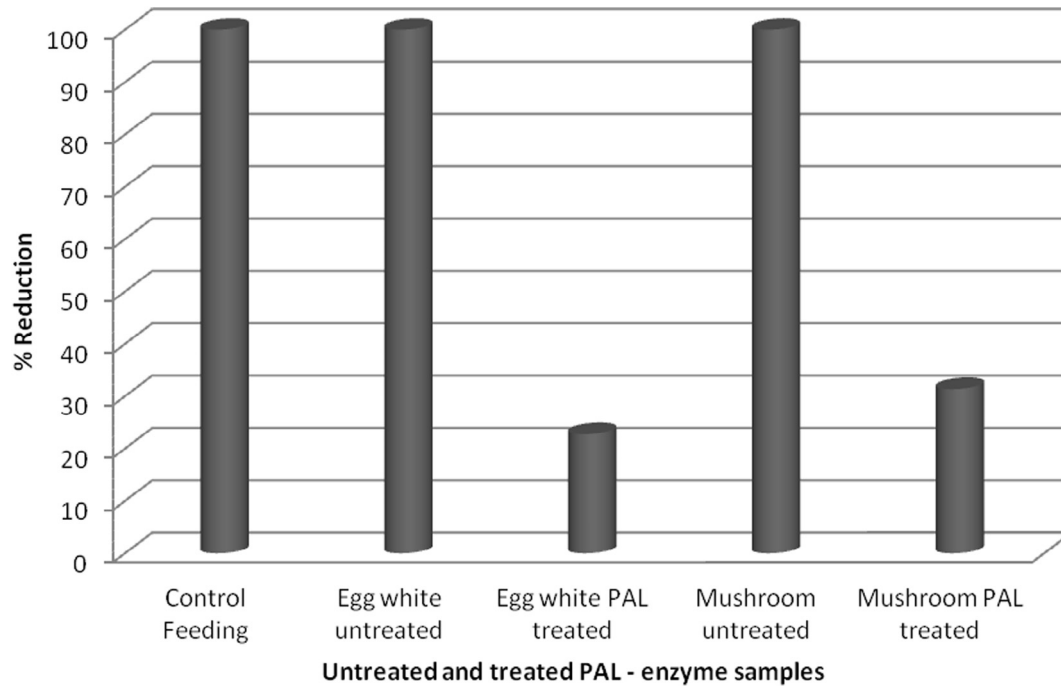| Treatments	Mice number	Phenylalanine (μmol/l)	ST. DEV	St. Error
Control	10	70.32	±11.90	±3.77
Egg white untreated	10	84.46	±14.13	±4.47
Egg white treated	10	65.23	±10.86	±3.44
Mushroom flour untreated	10	79.16	±5.15	±1.63
Mushroom flour treated	10	54.33	±14.13	±4.47

St. Error = SD/SQRT (n).

Fig. 3. Effect of PAL enzyme on the percentage reduction of phenylalanine concentration in blood mice feeding by egg white and mushroom flour with Tandem Mass method (n = 10).
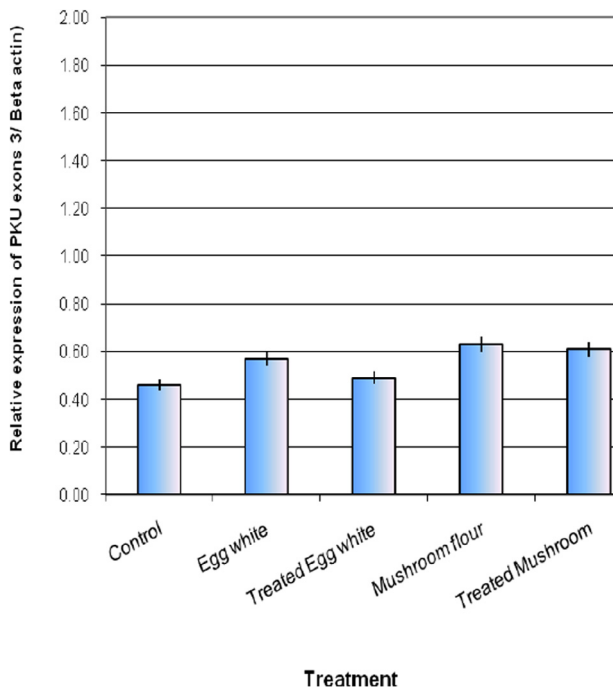

Fig. 4. The alteration of PKU exon 3-mRNA in brain tissues of female mice fed on diet contained untreated or treated egg white and mushroom flour. Data are presented as mean ± SEM. Mean values within tissue were insignificantly different ($P > 0.05$).
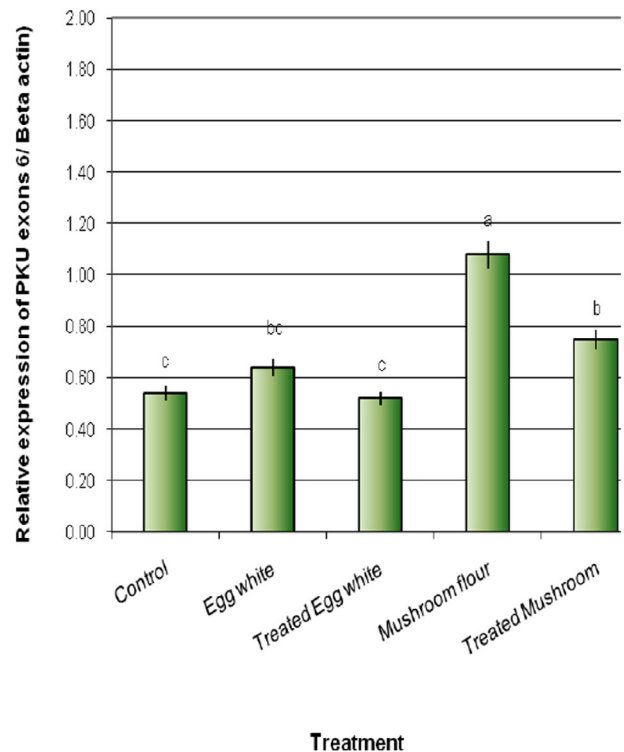

Fig. 5. The alteration of PKU exon 6-mRNA in brain tissues of female mice fed on diet contained untreated or treated egg white and mushroom flour. Data are presented as mean ± SEM. [a,b,c] Mean values within tissue with unlike superscript letters were significantly different ($P < 0.05$).

Concluded that the treated food with PAL extracted can reduce the phenylalanine level and open a new way to apply into different types of food for PKU patients to compensate the shortcomings in free phenylalanine formulas and decrease the compliances from PKU diets.

3.5. Expressions of PKU exons 3, 6, 7, 11, and 12 in female mice fed on diet contained untreated or treated egg white and mushroom flour

Figs. 4–8 represent the expressions of PKU exons 3, 6, 7, 11, and 12 in brain tissues of female mice fed on diet contained untreated
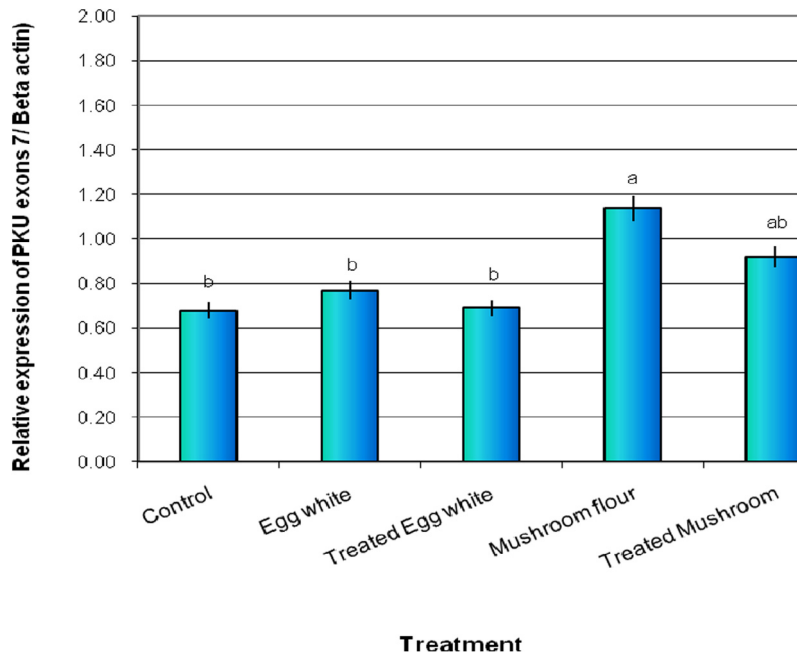
Fig. 6. The alteration of PKU exon 7-mRNA in brain tissues of female mice fed on diet contained untreated or treated egg white and mushroom flour. Data are presented as mean ± SEM. [a,b,c] Mean values within tissue with unlike superscript letters were significantly different ($P < 0.05$).
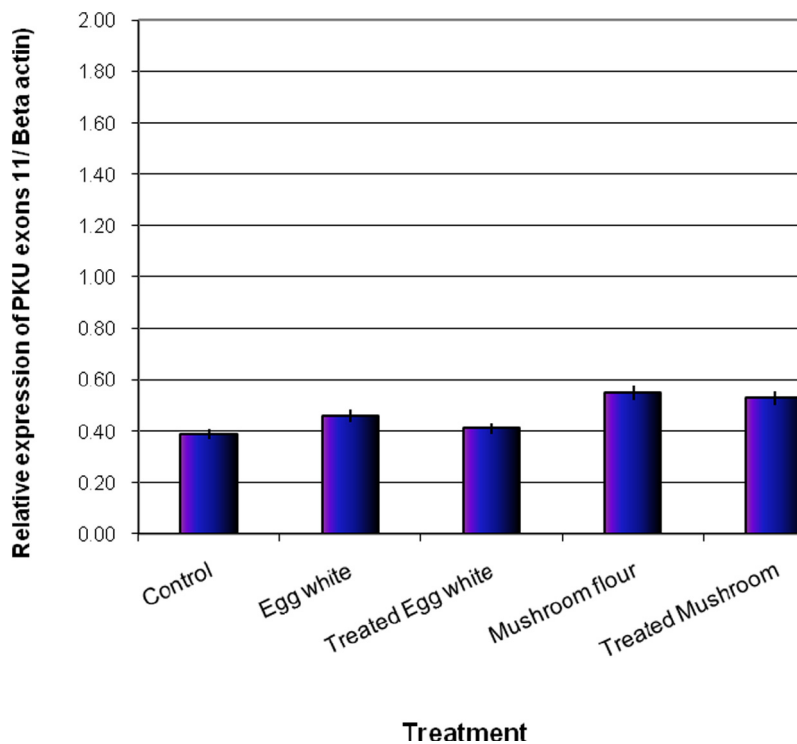

Fig. 7. The alteration of PKU exon 11-mRNA in brain tissues of female mice fed on diet contained untreated or treated egg white and mushroom flour. Data are presented as mean ± SEM. Mean values within tissue were insignificantly different ($P > 0.05$).

or treated egg white and mushroom flour. The results revealed that the expression levels of PKU exons 3, 6, 7, 11, and 12 were down-regulated in control mice fed on standard diet compared with female mice in other groups. In the same line, female mice fed on diet contained treated egg white exhibited relatively similar expression levels of PKU exons 3, 6, 7, 11, and 12 compared with those in control mice (Figs. 4–8). The expression levels of PKU

exons (3, 6, 7, 11, and 12) in female mice fed on diet contained untreated egg white were higher but without significant differences compared with control mice (Figs. 4–8). On the other hand, the expression levels of PKU exons were up-regulated in mice fed on diet contained untreated mushroom flour compared those in female mice fed on standard diet or those fed on egg white; however, these levels of expression were only significant in exons 6 and
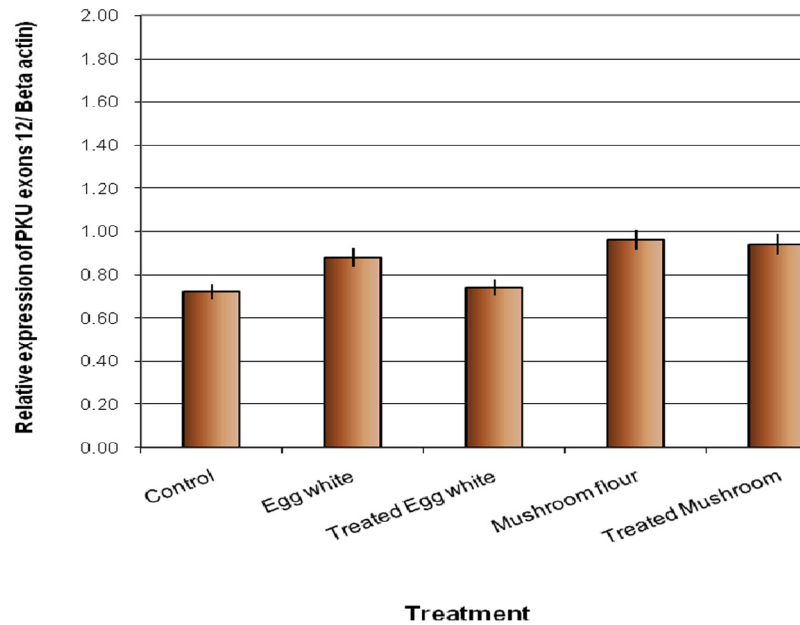
Fig. 8. The alteration of PKU exon 12-mRNA in brain tissues of female mice fed on diet contained untreated or treated egg white and mushroom flour. Data are presented as mean ± SEM. Mean values within tissue were insignificantly different ($P > 0.05$).
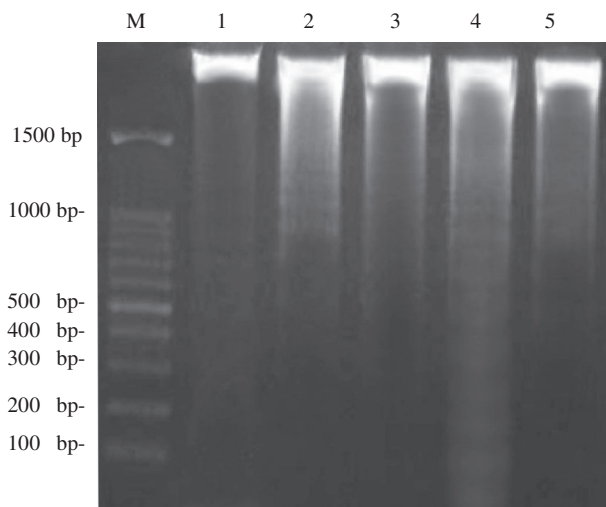

Fig. 9. DNA fragmentation in liver tissues of female mice fed on diet contained untreated or treated egg white and mushroom flour. M: DNA marker. Lane 1 represents PCR products of untreated control mice; lane 2 represents mice fed on egg white; lane 3 represents mice fed on treated egg white; Lanes 4 represents mice fed on mushroom four. Lane 5 represents mice fed on treated mushroom flour.

Table 3
DNA fragmentation in liver tissues of female mice fed on diet contained untreated or treated egg white and mushroom flour analysed by diphenylamine reaction procedure.

Treatments	% of DNA Fragmentation	
	Range	Mean ± SEM
Control	6–15	7.8 ± 0.2b
Untreated egg white	8–18	10.3 ± 0.3b
PAL treated egg white	7–16	8.2 ± 0.2b
Untreated mushroom flour	11–20	15.9 ± 0.4a
PAL treated mushroom flour	7–19	12.4 ± 0.3ab

7 (Figs. 5 and 6). In same line, female mice fed on diet contained treated mushroom flour exhibited higher expression levels of PKU exons compared with those fed on standard or diet contained egg white, while, these levels of expression were only significant in exon 6 (Fig. 5).

3.6. Effect of untreated or treated egg white and mushroom flour on the DNA fragmentation

The results of DNA fragmentation revealed that female mice fed on diet contained untreated or treated egg white and mushroom flour showed different rats of DNA fragmentation (Fig. 9 and Table 3). The rate of DNA fragmentation in female mice fed on diet contained treated egg white revealed similar rate of DNA fragmen-tation compared with control mice. However, the rate of DNA fragmentation in female mice fed on diet contained untreated egg white increased slightly compared with those in control mice (Table 3). On the other hand, supplementation of female mice with diet contained untreated mushroom flour increased significantly the rate of DNA fragmentation compared with those in control mice (Table 3). However, administration of female mice with diet contained treated mushroom flour increased insignificantly the rate of DNA fragmentation compared with those in control mice (Table 3).

Several findings suggested that several oxidative stress resources are correlated with the development and acceleration of hyperphenylalaninemia in PKU patients. Based on these results, Rocha and Martins [31] and Ribas et al. [29] suggested that increase the levels of Phe and/or its metabolites is coinciding with increase the reactive oxygen species and antioxidant status in the diets of PKU patients.

It has been reported that extreme generation of reactive oxygen species induce damage of the complete cell. The damage of the cell includes inhibition of the enzymes necessary for cellular processes, break of the DNA structure, and increase the apoptosis [15]. This damage attributed to the generation of reactive oxygen species could be suggested by oxidation of the DNA bases and break the linkage between the structure of protein and DNA [14]. I consistent of these literature, we have found that feeding of female mice with high source of phenylalanine diets (e.g. egg white and mushroom

flour diets) increased the expression alterations of PKU exons 6 and 7 as well as increase the rate of DNA fragmentation compared with mice fed on standard diet. The miss-binding between the structure of protein and DNA may lead to induce mutations, alteration in the gene expression, chromosomal abnormalities, rearrangement of the DNA structure, cellular-toxicity [9]. On the other hand, the present study showed that feeding of female mice with diets contained egg white or mushroom flour treated with PAL enzyme decreased the expression alterations of PKU exons 6 and 7 as well as declined the rate of DNA fragmentation compared with mice fed on a standard diet. The protective effect of PAL against high levels of phenylalanine is that PAL is an autocatalytic protein responsible to catalyse the phenylalanine suppressing its genetic toxicity functions in female mice.

4. Conclusion

According to the application of recent treatment tool several points should be taken into consideration such as the scientific doubts, potential risks, overheads, and sociological outcomes for the kids and their families. Based on this study the addition of PAL enzyme in egg white and mushroom flour showed that improve colour characteristics, decrease phenylalanine concentrations and its genetic toxicity, which can be used and applied in different wide range of foods as a functional food for PKU patients.

Acknowledgements

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