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Enzymatic Preparation of Low-Phenylalanine Formula Derived from Skim Milk Hydrolysate for Phenyl ketonuric Patients

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ABSTRACT

Background: Phenylketonuria (PKU) is one of the most common inborn errors of amino acids metabolism. WHO guidelines introduced in 1979 and revised 1988 for breast-feeding infants with PKU included a formula containing low amounts of phenylalanine as a part of dietary prescription. Mental retardation can be prevented if PKU is diagnosed in the 1st three weeks of life and diet therapy started straightaway throughout life and especially in the hyper phenylalaninemic mothers before conception and during pregnancy.
Aim of the Work: The aim of the present study was to synthesize a low-phenylalanine formula suitable to be taken by PKU children, adolescents and the hyperphenylalaninemic mothers.
Materials and Method: This formula should be of high biological value, taken safely by those patients and to be of low cost. The formula was prepared from skim milk hydrolysate using two proteolytic enzymes. The first was the immobilized purified papain enzyme and the second was the modified protease XXIII prepared from Aspergillus oryzae. The skim milk hydrolysate was adsorbed on barium sulphate or activated carbon for removing phenylalanine. They were applied separately for the purpose of debittering and nutritional value comparison.
Results: This skim milk hydrolysate had been supplemented with the amino acids tryptophan, tyrosine, methionine and valine. Beside the comprehensive amino acids analysis (Especially for the free amino acids), this formula was then analyzed for protein, fat, lactose and ash contents as well as microbiological and biological testing on mice. Hyperphenylalaninemia was induced in BALB/c mice model then changes in blood phenylalanine level and weight were scored during the periods of mutagenesis as well as the treatment period compared with the control group.
Conclusion: The amino acids analysis showed that phenylalanine was 0.71gm/100 gram protein in the skim milk hydrolysate compared to 3.26gm amino acid/100 gram protein in the skim milk. The level of free phenylalanine decreased from 6.34% (In the skim milk) to 0% after adsorption to barium sulphate and compared to 3.41% after adsorption to activated carbon.

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The formula adsorbed on barium sulphate, although it is more preserving to the nutritional composition; yet, it is less effective in the debittering effect than that adsorbed on activated carbon. This formula, in addition to being of high nutrional value, it is not expensive since it is obtained from skim milk hydrolysate. From the present study, it could be concluded that: The synthesized low-phenylalanine formula was effective in supplying most of the needed dietary intakes for conditions of hyperphenylalaninemia. The use of the immobilized purified Papain and modified protease XXIII from Aspergillus oryzae in enzymatic hydrolysis of skim milk has been proved to be effective in hydrolysis and emulsification.

Key Words:

Phenylketonuria, skim milk.

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INTRODUCTION

Breast milk is the only food that absolutely fulfills the nutritional and immuno-protective requirements of neonates and babies of all mammals. Human milk is consequently considered the best source for infants because of its components that play multiple roles in growth of infants and young babies and keeping them healthy.[1] In some circumstances, however, bottle-feeding is a must as a substitute for mother's milk.[2] Phenylketonuria (PKU; Mckusick 261600) is one of such cases.

Deficiency of phenylalanine hydroxylase results in failure of normal phenylalanine metabolism that leads to accumulation of phenylalanine and its abnormal metabolites in the blood, all of the body tissues and the brain causing severe mental retardation.[3] On the other hand, dietary noncompliance in pregnant women suffering hyperphenylalaninemia can produce developmental abnormalities and mental impairment in the offspring.[4] The only practical mode of treatment is giving the phenylkutonuria patients a low-phenylalanine diet.[5] The phenylalanine content of various protein sources has been reduced by adsorption chromatography[6], organic solvents or industrially unsuitable chromatographic media.[7] In formulae synthesis, the use of purified immobilized enzymes has been reported to induce more stable preparation with higher degree of hydrolysis without microbial growth.

This work was undertaken to establish a feasible protocol for removing phenylalanine from skim milk using enzymatic hydrolysis by purified immobilized enzymes aiming to achieve medically safe milk for such infants with PKU and pregnant patients with hyperphenylalaninemia, with the least loss in milk nutritional value. A low-cost protocol and easy assay technique were also targeted.

MATERIALS AND METHODS

Materials:

1. Dry skim milk was reconstituted by dissolving it in demineralized water

(Milli-Q water prepared with Millipore system and filtered on a 0.22µl filter Millipak) at a ratio of 1:9.

2. Two enzymes were used in the enzymatic proteolytic process; the 1st was protease type XXIII from aspergillus oryzae (Cat# P-4032, Sigma chemical Co, USA), and the 2nd was Papain immobilized and purified enzyme (Cat# P-4406, Sigma chemical Co. USA).

3. Adsorption materials: activated carbon powder and barium sulphate powder are used for removing phenylalanine from the hydrolysate.

4. Supplements of amino acids (According to the FAO/WHO provisional pattern): Methionine (2.29mg/gm protein), Tryptophan (0.96mg/gm protein), Valine (4.96mg/gm protein) and Tyrosine 92.45mg/gm protein) (Sigma chemicals Co. USA).

5. Supplements of vitamins and minerals in amounts according to the FAO/WHO provisional pattern (Supplied by Sigma chemicals Co, USA).

Methods:

1. Skim milk hydrolysates were prepared by two enzymes: purified Protease XXIII and the immobilized purified Papain enzyme[9] in a 10% total solids solution. Hydrolysis processes were applied in a water bath equipped with mild shaking and temperature control capabilities. Concentrations of the enzymes were expressed as a percentage of the substrate and the digestion was performed according to the original method described by Clegg and McMillan[10] and later on by Lopez–Bajonero et al.[11] Hydrolysis was stopped by heating the mixture for 10 minutes at 92°C then hydrolysate was cooled to -20°C then the two enzymes were applied. For adsorption process, the hydrolysed skim milk was shaken with 200% activated carbon vs. protein for 2 hours at room temperature or barium sulphate 200% vs. protein for 1 hour at 40°C. Once the separation process was achieved, the mixture was vacuum filtered. After filtering off the adsorbents, the hydrolysates were subjected to sensory analysis. The clear solution was concentrated to 25% solids by rotary evaporator. Both hydrolysates were stored at -20°C. The final product was examined for dry matter; total protein, non protein nitrogen, fat, ash and lactose (American Official Analytical Chemists).[8]

2. Protease activity was assayed by the method originally described by McDonald and Chen.[12] The substrate used was 1% (W/V) casein solution buffered at pH 7.5 using phosphate buffer. To 1ml casein solution, 1ml of the enzyme preparation was added and incubated at 3°C for 20 minutes. Three ml of 5% (W/V) trichloroacetic acid was then added and left to stand for 30min. The precipitated protein was separated by filtration. Then 1ml of filtrate was added to 3ml of 3.75% (W/V) Na_2CO_3 then after 10min., 1ml of Folin reagent 1:3 dilution was added. The mixture was allowed to stand for 30min. at 30°C and the absorbance was then measured at 660nm. Standard curve was

plotted and enzyme activity was expressed as U/g tyrosine released from casein at 30°C. Specific activity was defined as units of enzyme/ mg of protein.[13]

3. Degree of hydrolysis was determined by precipitating the hydrolysate in 12% trichloroacetic acid. The nitrogen content of the supernatant (NPN) was measured by micro-Kjeldahl method as follows : D.H. (%)=NPN/total N x 100[14]

4. Adsorption process of the hydrolysate was performed on activated carbon as described by Hassler[15] or on barium sulphate as described by Helbig et al.[16]

5. Amino acid analysis performed on amino acid analyzer LC3000 (Pharmacia - Amercham, Switherland). HCl hydrolysis was performed prior to amino acid derivatization using 6N HCl for 21 hr at 110°C. Protein determination was done where nitrogen content of the samples was determined by the micro-Kjeldahl method.[8]

6. Determination of fat content was based on the original modified Gerber method.[17]

7. Mineral measurements: was determined using atomic absorption spectrometry (Hitachi Ltd., Tokyo, Japan) according to the method of Morr and Foegeding.[18]

8. Moisture was determined by drying at 105°C using air oven to have constant weight according to AOAC.[8]

9. Lactose analysis according to the original test of Dubois, et al.[19]

10. Ash was determined by incinerating the dried samples at 550°C to constant weight according to AOAC.[8]

11. Microbiological assay for safety of the formula using total plate count[20], detection of coliform group of microorganisms using lactose broth medium[21] and also detection of yeast and molds using malt extract agar medium[8] acidified to PH 3.5 and incubated at 30°C for 3 days then the colonies were counted.

12. Sensory analysis: The bitterness of 10% of aqueous treated skim milk hydrolysate was scored on a 9-point Hedonic Scale.[22]

13. Biological feeding and evaluation on BALB/c mice (Both hyperphenylalaninemic and normal control) as follows:

13-1. Animal feeding experiment:

Sixty-six (66) adult female BALB/c mice (3 weeks of age) were fed in the experimental animal house of the Research Institute of Ophthalmology (Giza), under normal healthy conditions for one week on a normal basal diet which contains salt mixture (4%), vitamins mixture 1%, 10% cellulose, 65% starch, 10% corn oil and 10% protein.[8] At the end of the adaptation period, mice reached an approximate weight of 20-25gm. The animals were housed in cages at room temperature (About 25±5°C).

13-2. Design of experiment:

After one week (Adaptation period),

the mice were divided into two groups, normal mice (18) and future mutagenic mice.[48] The experimental diet and tap water were supplied adlib. Total body weight of the animals was recorded at the beginning and the end of the experiment.

13-2.1. Normal BALB/c mice (Control):

Eighteen (18) BALB/c mice (4 weeks of age) were divided randomly into three experimental groups, each consists of six mice with weekly measurement of blood phenylalanine and mice body weight; according to the following scheme:

Group 1: Fed on basal diet for 28 days.

Group 2: Fed on hydrolysate adsorbed on activated carbon for 28 days.

Group 3: Fed on hydrolysate adsorbed on barium sulphate for 28 days.

13-2.2. BALB/c mice phenylketonuria model:

The stage of induction of mutagenesis lasted for 21 days. Forty-eight (48) normal BALB/c mice (4 weeks of age) were fed on 10gm of mutagenic basal diet containing L-ethionine (0.3% w/w) and L-phenylalanine (3% w/w). The rate of phenylalanine and ethionine consumption was fixed to about 1.14 gm/week.[23] Experimental animals receiving the combination of L-ethionine and L-phenylalanine developed hyperphenylalaninemia after 2 to 3 weeks of dietary regimen. Every week, total body weight and mutagenesis material consumed were scored and phenylalanine level in blood was determined in blood samples withdrawn under diethyl ether anesthesia from retrobulbar venous plexus of randomly chosen six mice according to the procedure of Schermer.[24]

13-2.3. Phenylketonuric mice under dietary treatment:

After three weeks period (Mutagenic period), eighteen[18] BALB/c mice (7 weeks of age) chosen from the mutagenesis group and were divided randomly into three experimental groups, each consists of six mice with weekly measurement of blood phenylalanine and mice body weight; according to the following scheme:-

Group 1: Fed on basal diet for 28 days (Unrestricted diet)

Group 2: Fed on hydrolysate adsorbed on activated carbon for 28 days.

Group 3: Fed on hydrolysate adsorbed on barium sulphate for 28days.

13-3. Phenylalanine level:

Phenylalanine level was estimated in blood of mice in zero time and every week of the mutagenic period (21 days) and during the experimental feeding period (28 days) according to the modified procedure based on the method of McCaman and Robins[25] using a HPLC equipment Wallac fluorometer system TM (Perkin-Elmer Life Science Inc., Norton, OH, USA). The assay is based on the enhancement of the fluorescence of a phenylalanine-ninhydrin reaction product by the dipeptide, L-leucyl-L-alanine. This method measures phenylalanine quantitatively in the presence of other amino acids.

RESULTS AND DISCUSSIONS:

Table (1) shows the amino acid composition in skim milk and skim milk hydrolysate. These data revealed that the phenylalanine was 0.71gm% in the

skim milk hydrolysate compared to 3.26% in the skim milk respectively. The amounts of aspartic acid, glycine and histidine were much higher in skim milk than the skim milk hydrolysate. The other amino acids were all less detectable in the skim milk. The tyrosine content was 3.68gm% in skim milk hydrolysate compared to 2.65gm% in skim milk.

Table 1: Amino acid composition* in skim milk and skim milk hydrolysate.

Amino acid	skim milk	Skim milk Hydrolysate
Alanine	3.1	3.12
Arginine	1.4	2.11
Aspartic acid	8.11	6.18
Cystine	3.45	4.54
Glutamic acid	13.62	14.72
Glycine	4.02	2.88
Histidine	10.28	3.35
Isoleucine	6.34	8.22
Leucine	7.81	7.91
Lysine	4.71	7.49
Methionine	1.91	3.85
Phenylalanine	3.26	o.71
Prolin	13.09	13.48
Serine	4.71	5.86
Thereonine	2.64	4.72
Tyrosine	2.65	3.68
Valine	1.76	1.97

* gm amino acid/100 gm protein.

** ND = not detectable.

Table (2) shows the levels of free amino acids in skim milk hydrolysate before and after adsorption. The level of phenylalanine was 6.34% in the skim milk hydrolysate before adsorption while it disappeared in the formula adsorbed on barium sulphate and decreased to 3.41% in the formula adsorbed on activated carbon. The tyrosine was 1.07% in the barium sulphate-adsorbed formula and 1.81% in the activated carbon-adsorbed formula compared to 5.09% before adsorption and 0.67% in skim milk. The tryptophan was 3.77% in the formula adsorbed on barium sulphate. The histidine was not detectable in the barium sulphate formula compared to 1.31% in the skim milk sample. On the other hand, neither tryptophan nor phenylalanine was detectable in the skim milk sample.

Lopez Bajonero et al.[11] stated that after phenylalanine separation on activated carbon, some phenylalanine remain in the form of low molecular weight peptides rather than in the free amino acid form. Tyrosine and tryptophan behave similarly to phenylalanine. So, selection of carbon level must be adjusted. In general, the design criteria for carbon adsorption process were found to be 2.95gm of carbon per gm casein.

In the present study, the milk as a natural source of protein hydrolysate was used to obtain a low phenylalanine formula.

This is in agreement with Mira et al.[26] who recommended the use of dietary alternatives to the synthetic amino acids mixtures free of phenylalanine and based on low phenylalanine protein hydrolysate.

In the present study, skim milk hydrolysate was used for synthesis of low-phenylalanine formula and the findings of amino acid patterns in this study are in agreement with Boehm et al.[27] who found that infants fed on whey fortifier had significantly higher threonine concentration compared to those fed exclusively human milk protein whereas levels of some other essential amino acids (Valine, leucine, lysine, histidine,

Table 2: Free amino acids composition* in skim milk and skim milk hydrolysate before and after adsorption on activated carbon and barium sulphate (%).

Free Amino acid	Skim milk	Skim milk hydrolysate		
		Before Adsorption	After Adsorption	
			Activated carbon	Barium sulphate
Alanine	1.81	2.11	3.58	7.13
Argnine	17.18	4.83	1.09	7.52
Aspartic acid	ND**	8.46	9.75	7.87
Cystine	ND**	5.13	2.43	ND**
Glutamic acid	13.16	18.87	16.91	14.06
Glycine	1.11	2.17	4.48	6.05
Histidine	1.31	ND**	6.64	ND**
Isoleucine	1.4	2.93	7.36	6.87
Leucine	2.43	10.48	ND**	7.8
Lysine	8.18	5.04	3.46	10.93
Methionine	1.11	3.69	2.28	2.09
Phenylalanine	ND**	6.34	3.41	ND**
Prolin	ND**	8.08	18.51	4.17
Serine	3.32	2.82	6.2	4.75
Threonine	6.08	2.43	4.29	5.02
Tryptophane	ND**	ND**	ND**	3.77
Tyrosine	0.67	5.09	1.81	1.07
Valine	3.13	ND**	1.9	8.77

* gm amino acid/100 gm free amino acids.
** ND = not detectable.

phenylalanine and tryptophan) were all lower in concentration. Darling et al.[2] stated that infants fed casein dominant formulae have higher plasma phenylalanine and tyrosine concentrations than those fed mothers' milk. Conversely, elevated plasma threonine concentrations are observed in infants fed-whey dominant formulae.

Benson et al.[3] stated that, the goal in preparation of infant formula would be to imitate the protein or amino acids composition of human milk without causing metabolic stress. The amino acid is essential if it is demonstrated to promote positive nitrogen balance. In addition to the classic list of essential amino acids, other amino acids are necessary for the developing infants. Second group of amino acids known as semi essential, conditionally essential, or developmentally essential amino acids. Cysteine, tyrosine, histidine and glycine have been proposed for inclusion in this list.

The nutritive value of protein mostly depends on its amino acids profile in general and the quantities of essential amino acids in particular. Protein requirements can be satisfied by providing the essential amino acids rather than by increasing the total intake of protein.[28]

Karagoz et al.[29] stressed on the importance of the essential amino acids intake and the nature of amino acids pattern in

diet. The authors stated that restricted essential amino acids intake and altered pattern of plasma amino acids and plasma protein levels together with the trace elements have adverse effects especially on humoral and cellular immune functions.

The aim of attaining high glutamic acid level for PKU patients in the skim milk hydrolysate formula prepared in this study is important in light of the finding by Neu et al.[30] who stated that, a glutamine enriched formula shows evidence of improved tolerance to enteral feeding and situations with sepsis requiring medical intervention.

In the present study, the following amino acids were added to the skim milk hydrolysate as a supplementation:- methionine, tryptophan, valine and tyrosine (According to the recommended amount of FAO/WHO provisional pattern).

Smith et al.[31] reported a sodium independent carrier system that transports the large neutral amino acids (LNAAs) i.e. (Valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, methionine, threonine and histidine) across the blood brain barrier. Those amino acids may affect behavior. In rats, an increase in brain tryptophan causes increase in the brain serotonin which had good effect on sleep rhythm, pain sensitivity and mode of aggression. Also in support for our results, Matalon et al.[32] stated that large neutral amino acids lowered brain and blood phenylalanine levels in mice with PKU. This finding is explained as phenylalanine has the lowest Michaelis constant (Km), so, increasing other LNAAs competes with the transport of phenylalanine into the brain through shared carrier.

Austic et al.[33] stated that the branched chain amino acids have been reported to improve fetal brain development in PKU rats model and that large neutral amino acids (LNAAs) supplementastion to the synthetic diet improved fetal amino acids profiles in maternal PKU conditions.

Brass et al.[34] stated that the fetal cerebral concentrations of methionine and branched chain amino acids (Valine, leucine and isoleucine) were decreased by hyperphenylalaninemia.

The PKU diet formula should be a low possible protein nutrition with supplementation of tyrosine.[35] In PKU, without restriction of dietary phenylalanine intake, phenylalanine and its metabolites accumulate in blood and tissues and tyrosine becomes deficient. Therapy should consist of restricting dietary phenylalanine to an amount that results in near normal plasma phenylalanine concentration and tyrosine is supplemented in an effort to maintain normal plasma tyrosine concentration.[36]

Joseph and Dyer[35] found that when an eight–fold elevated blood phenylalanine decreased to near control levels, the blood tyrosine gradually rose to near normal values. However, in the brain frontal cortex and striatum, tyrosine levels increased but remained less than 70% of the control.

Our skim milk hydrolysate preparation contained higher amount of isoleucine than FAO/WHO recommendations (8.22 gm% vs.4%) and more leucine than the FAO/WHO patterns (7.91% vs. 7.04% respectively) and was further supplemented with methionine and valine in light of the finding by Austic et al.[33] that in hyperphenylalaninemic rats

there is lower concentrations of isoleucine, leucine, valine and methionine.

Also, supplementation of the diet of gestating rats with mixture of LNAAs ameliorates the adverse effects of increased phenylalanine on fetal brain growth and results in markedly lower phenylalanine concentration not only in fetal brain but also in fetal and maternal blood. The same results were obtained in human where dietary supplementation with LNAAs has been shown to lower brain phenylalanine concentration in adults with PKU.[37]

In PKU rats, the concentration of threonine was found to be high and that of glycine markedly elevated in brain when the rats were fed increased phenylalanine diet, therefore, it seems desirable to omit threonine from the amino acids supplementation since threonine also is a precursor of glycine.[33]

As shown in Table (3), skim milk has higher protein content (31.7gm/100gm dry matter) than skim milk hydrolysate (25.29gm/100gm dry matter) but lower fat, lactose and ash. The degree of hydrolysis was much higher for the skim milk hydrolysate (83.95%) than for the skim milk (10.15%), the non protein nitrogen (N.P.N) was higher in skim milk hydrolysate by about 7 folds, while the total nitrogen was higher in the skim milk (31.7%) than the skim milk hydrolysate (25.29%).

Boza et al.[38] stated that weight gain is associated with statistically higher nitrogen retention. Mahmoud et al.[39] stated that extensive hydrolysis is essential to render the protein immunologically unreactive. When producing hydrolyzed proteins, it is important to determine the degree of hydrolysis. Our results are also in agreement with Boza et al.[40] who reported that N retention was higher in rats given the protein-hydrolysate-based diets compared to those given the intact-protein-based diets. This was associated with a lower urinary N excretion. It has been found that enzymatic hydrolysis of milk protein has equivalent effects to native protein on recovery after starvation in rats and even gives higher N retention with same gain in body weight and digestibility without any noticed harmful effects.

In the present study, we used immobilized purified proteolytic enzymes for enzymatic hydrolysis based on the finding of Ge et al.[41] that for the immobilized papain, Km (app) was similar to that observed with soluble papain. Also, it is stable when stored at 4°C and pH 7.5 for periods up to 8 months whereas soluble enzymes lose their activity within 96 hours under similar storage conditions. Immobilized papain did not lose any activity after treatment with 6M urea for 270 minutes whereas soluble papain lost 81% of its activity due to altered structural and conformational

Table 3: Degree of hydrolysis (DH) and composition of skim milk and skim milk hydrolysate (gm/100gm dry matter).

Sample	Protein (T.N)	N.P.N %	DH %	Lactose	Fat	Ash
Skim milk	31.7	3.22	10.15	50	0.9	7.5
Skim milk hydrolysate	25.29	21.23	83.95	54	1.2	8

stability. The specificity of protease is of crucial importance for both the degree of hydrolysis and the free amino acids content of the hydrolysate. The immobilized Aspergillus Oryzae protease was the most effective enzyme in breaking down casein molecules and releasing the free amino acids from casein hydrolysate. It can be used for preparing infant formulae, and as an ingredient for food industry.[41]

Kilara and Shahani[9] and Lee et al.[42] and Tardioli et al.[43] recommended inducing hydrolysis by immobilized enzymes.

Ambrus et al.[44] reported the use of enzyme reactors with immobilized enzymes for phenylalanine depletion for the management of PKU. The procedure was reported to be rapid and sustained. Immobilized enzymes are not metabolized to a significant degree and thus small amounts can be used repeatedly.[44,45]

In our study, the degree of hydrolysis (DH) was (83.95%) using combined papain and protease XXIII enzymes.

This is in consistency with Lopez–Bajonero et al.[11] who induced enzymatic hydrolysis of skim milk powder by using two enzymes, protease 2A (5 hours) from the Aspergillus oryzae followed by papain hydrolysis (21 hours) to liberate 16.5mg phenylalanine per gm protein. Cogan et al.[46] used papain and pepsin; Dubois et al.[19] used pronase and ficin; Arai et al.[7] tested a series of enzymes with pepsin-pronase mixture; while Mannheim and Cheryan[47] used continuous hydrolysis of milk protein in a membrane reactor using alcalase. Proteases being degradative enzymes that catalyze total hydrolysis of protein, account for about 60% of the total world wide sale of enzymes.[48]

Table 4: Minerals content of skim milk, skim milk hydrolysate treated with activated carbon and skim Milk hydrolysate treated with barium sulphate (ppm).

Minerals	Skim milk	Hydrolysate treated with activated carbon	Hydrolysate treated with barium sulphate
Ca	39.81	30.471	24.762
Zn	0.576	0.375	0.661
Mg	0.744	0.5	0.449
Fe	0.73	0.66	0.26
Mn	0.066	0.049	0.061
Na	1.72	1.019	1.309

In the present study, vitamin A and Ergocalciferol were also added as supplementation to the formula. The content of Zinc was highest 0.661 ppm in the hydrolysate treated with barium sulphate as compared to 0.576 pmm in the skim milk and only 0.375 pmm in the formula treated with activated carbon.

Bushueva et al.[49] demonstrated the necessity of diet correction by means of β-carotene containing vitamin A supplementation. Individuals with PKU have been reported to have altered trace mineral status. PKU mice on unrestricted diet had lower hepatic zinc level and a greater concentration of hepatic iron by 1.5 times as compared to the control group of mice.[50,51]

Hyperphenylalaninemia adversely affects bone status and osteocalcin in PKU mice.[52]

Fisberg et al.[53] stated that PKU-formulae must supply trace elements and vitamins that are usually supplied by whole protein foods. The authors also stated that plasma zinc of PKU children≥7years was significantly lower than normal control children.

Acosta[54] found that when L-amino acids were the source of protein to PKU infants, their plasma retinol concentrations were marginal or deficient. The authors attributed the cause to be the restricted intake of high biological value protein. Karagoz et al.[29] stated that since all patients with PKU have zinc deficiency, they should be supplemented with zinc regularly.

Hogan et al.[55] emphasized the importance of records of diet intakes especially for total protein and vitamins (D, E,A, ascorbic acid and B-complex group) to be adequate in PKU patients on phenylalanine restricted diets.

Matalon et al.[32] clarified the importance of medical food supplementation especially for women with PKU on medical food where protein intake is low; therefore, vitamin and mineral intake could be subsequently inadequate.

In the present study, amino acids pattern was more preserved with the use of barium sulphate (BaSO4) but bitterness depletion was achieved more by adsorption on activated carbon.

This is in agreement with Dubois et al.[19] who compared various adsorption methods for debittering skim milk hydrolysates. They found that, bitter peptides were mostly hydrophobic as they were almost completely eliminated by hydrophobic chromatography on hexylepoxy sepharose. Activated carbon was the most effective in adsorbing bitter peptides. The inorganic adsorbents barium sulphate ($BaSO_4$) and Fuller's earth exhibited negligible bitter peptide adsorption capacity as did the plastic low density polyethylene and polyvinylidene chloride. However, analysis of activated carbon-treated skim milk hydrolysate revealed 60% decrease in riboflavin and 3% decrease in protein without any change in lactose, calcium and Ash content. Essential amino acids analysis indicated that the levels of leucine, isoleucine, valine and total aromatic amino acids dropped during treatment to 81% of the reference values.[19]

Microbiological assay was done to ensure that sterility was maintained during sample preparation, so; standard

count, coli form, mold and yeast testing were performed. After storage, the total bacterial count as well as yeast and mold counts were 13x102 per gram and 37x101/gm respectively. On the other hand, coli forms were not detectable in any of the prepared samples.

Marero et al.[56] recommended that for infant formulae to be microbiologically safe for consumption, it should be free from coliforms and might have low total plate count (TPC) at max.(10^2).

Brindisi et al.[57] stated that sterility should be maintained during sample preparation through standard plate counts (SPC) and coliform tests.

The Egyptian Organization for standardization and quality control necessitates that total bacterial count should not be more than 1000 organisms/gm for infants' food which is prepared without boiling. Coliform group should be less than 10 organisms per gram food which is prepared for use by boiling for 3 minutes or less and should be free of *E.coli*.

As shown in Table (5) the cumulative increase in phenylalanine level was accompanied by progressive decrease in weight of mice (25.54% in 1st week up to 47.55% by end of 3rd week of mutagenic period). The rate of weight loss was highest from day 0 to day 7 (7.45gm; 25.54%) and least from day 14 to day 21 (2gm only; 11.5%).

Because of the ethical aspects of withholding dietary treatment from patients with PKU, effectively studying long-term effects as post natal growth rates is difficult or impossible. The mutant mice animal model for human PKU, provides an opportunity to conduct studies to clarify these issues.[58]

Schott et al.[23] stated that valid animal models became available when the phenylalanine hydroxylase inhibitors as p-chlorophenylalanine and alpha-methyl phenylalanine were introduced. The female adult NMRI mice were used as a PKU model with 0.3% ethionine and 3% phenylalanine as PAH inhibitors. BALB/c mice and some other strains of rats have been used as suitable animal models to study PKU in many other studies by Brass et al.[34], Yannicelli and Medeiros[52]; Joseph and Dyer[35] and Gropper et al.[51]

The level of blood phenylalanine in surviving mice increased in a cumulative manner about 3.75 folds ranging from 1.327mg/dl (day 0) to 4.976mg/dl

Table 5: Effect of the mutagenic diet containing 3% L-phenylalanine and 0.3% L-ethionine on mice body weight, percentage of surviving mice and concentration of phenylalanine in blood for 21 days.

Day	Amount of phenylalanine and ethionine consumed (gm/week)	Mean values of phenylalanine in blood (mg/dl)	Weight {gm)	Loss of weight %	Surviving mice
0	0	1.327	29.17	0	100
7	1.144	1.621	21.72	25.54	100
14	2.2885	3.964	17.3	40.69	82
21	3.433	4.976	15.3	47.55	56

(day 21). The highest increase in level of phenylalanine existed between day 7 and day 14 (2.445 folds), while the rate of increase in level of phenylalanine in 1st and 3rd weeks was similar (1.2 fold increase). On the other hand, the highest value of phenylalanine among those died mice reached up to 10.314 mg/dl by the end of mutagenesis period.

Villa Trevino et al.[59] found that applying high dosage of ethionine together with phenylalanine additionally resulted in up to 57-fold increase in the concentration of phenylalanine.

Alpha-methylphenylalanine reduces the phenylalanine hydroxylase activity of rat liver by 75%. Daily injection and supplementation of this substance (Plus phenylalanine) to rats from 3rd to 15th day of age had no toxic effects and maintained a plasma concentration of phenylalanine comparable to that of phenylketonuric subjects.[60]

Schott et al.[23] demonstrated that applying ethionine together with phenylalanine resulted in hyperphenylalaninemia and PKU through inhibiting activity of phenylalanine hydroxylase and that ethionine and 3% phenylalanine are useful test substances for studying regulation of phenylalanine hydroxylase in vivo as well as a pharmaclogic model for phenylketonuria. The underlying mechanisms might be disturbed protein synthesis and protein phosphorylation through ethionine–induced suppression of phenylalanine hydroxylase. However, a percentage of phenylalanine in diet higher than 3% is not recommendable because of the so called amino acid toxicity of phenylalanine and that the hyperphenylalaninemic condition induced by application of a high dosage of ethionine and phenylalanine induced severe loss of body weight. McDonald[58] reported that reduced postnatal growth is an abiding feature of phenylketonuria in the mutant mouse model.

As shown in Table (6), the level of blood phenylalanine was nearly the same among all groups of mice after induc-

Table 6: Mean values of blood phenylalanine among all groups of mutagenesis mice model before start of treatment (Day 0) and during the feeding period (7, 14, 21, 28 days) compared with non mutagenesis mice in all groups (Control).

Group of mice	Mean values of blood phenylalanine (mg/dl)					Change in phenylalanine level (%)
	Day 0	Day 7	Day 14	Day 21	Day 28	
Basal diet feeding	4.951	5.18	5.347	5.545	5.941	+20%
Control	1.371	1.426	1.522	1.559	1.581	+15.46%
Activated carbon formulae	4.986	4.612	3.842	2.845	1.698	-65.94%
Control	1.328	1.236	1.03	0.803	0.502	-62.2%
Barium sulphate formulae	4.972	4.475	3.6	2.557	1.292	-74.01%
Control	1.341	1.218	0.969	0.698	0.376	-71.96%

tion of mutagenesis and before the start of feeding period (Day 0). There was no significant statistical difference between any of the 3 mice groups (P>0.05) with the blood phenylalanine values ranged between 4.951 and 4.986 mg/dl.

The levels of phenylalanine remained high in all mice groups at day 7 of feeding compared to the control groups. At day 14 of feeding, the mean level of phenylalanine increased by about 7.99% in the group of mice fed on basal diet but for the other 2 groups of feeding, there was 27.59% decrease in the mice fed on the formula treated by barium sulphate. The decrease was less in the group fed on the formula adsorbed on activated carbon (22.94% decrease). However, there was no significant statistical difference (P>0.05).

After 21 days of feeding, all the mice groups showed normal levels of phenylalanine except for the group fed on basal diet which increased by 11.99% (5.545mg/dl). The decrease in phenylalanine level in the group fed on barium sulphate-treated formula was 48.57% compared to only 42.94% decrease in the group fed on the activated carbon-treated formula.

By the end of day 28, the mean values of phenylalanine were normalized in all the formulae-fed groups; being lowest in the mice fed on bariun sulphate-treated formula (1.292 mg/dl; 74.01% decrease) and their control group (0.376 mg/dl; 71.96% decrease).

As shown in Table (7), the group of mutagenesis mice fed on the formula treat-

Table 7: Follow-up table for the mean of weights, percentage of weight changes and percentage of surviving mutagenesis mice during the feeding period among all feeding groups

Group of mice	Feeding period (Day)	Mean weight (gm)	% of initial weight	Surviving mice %
Basal diet feeding	0	15.3	100	100
	7	14.55	95.09	40
	14	13.35	87.25	40
	21	12.45	81.37	40
	28	11.4	74.51	20
Activated carbon -treated formula	0	14.74	100	100
	7	15.067	102.22	80
	14	15.23	103.32	80
	21	15.53	105.36	80
	28	15.97	108.34	40
Barium sulphate -treated formula	0	15.7	100	100
	7	16.17	102.99	80
	14	16.6	105.73	80
	21	16.95	107.96	80
	28	17.93	114.2	80

ed by barium sulphate showed an increase in weight accounting for 114.2% of the initial weight, followed by the mutagenesis mice fed on activated carbon-treated formula (108.34%) ($P>0.05$). On the other hand, the group fed on basal diet showed decrease in body weight about 74.51% of the initial weight.

Percentage of mice remaining alive was highest in the group fed on the formula treated by barium sulphate (80%) compared to only (20%) in the group fed on basal diet. About 20% of the experimental mice were lost in each of the feeding formula groups during the 1st week of treatment period but the group fed on activated carbon lost additionally about 40% by the 4th week of treatment period.

On the other hand, the feeding on basal diet increased the blood level of phenylalanine with increasing the mortality rate of mice.

This is in agreement with the findings of Josef and Dyer[35] who had placed phenylketonuric mice model on a low-phenylalanine diet for 4 weeks so as to drop blood phenylalanine levels from an eight–fold to normal control levels.

On the other hand, Yannicelli and Medeiros[52] studied 54 male weanling PKU and control mice and assigned them to either elemental phenylalanine-restricted diet (Treated) or phenylalanine-unrestricted diet (Untreated) with low or normal protein levels for 56 days. The results were analyzed at baseline, at day 28 and on day 56.

Delvalle and Greengard[61] found that maximum increase in plasma phenylalanine on day 15 of mutagenesis was accompanied by about 2% inhibition of brain and body growth.

To conclude, the technique of enzymatic hydrolysis could be the most useful alternative for preparation of low phenylalanine formulae, not only because of the economic advantage, but also because of the minimal loss of nutritional value of milk compared to the general amino acids damage that may result from other methods e.g. alkali and acid treatment. These formulae may be helpful for dietary management of PKU patients as well as mothers with hyperphenylalaninemia. The BALB/c mice have been proved to be a suitable animal model to study phenylketonuria disease. The use of 3% phenylalanine and 0.3% ethionine for 21 days was proved to be effective for induction of hyperphenylalaninemia in mice model.

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