Egypt. J. Chem. Vol. **68**, No.11, pp. 635- 641 (2025)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Approaches for enzymes behavior on phenylalanine removal from different dairy protein sources

Manar M. Abd El-Rahman [a*] , Nagwa M. Sidkey [b], Aziza H. Elkassaby [b], Nayra Sh. Mehnna [a],

[a] *Dairy Department, National Research Centre, 33 El Bohouth St. (former El Tahrir St.), Dokki, Giza P.O. 12622, Egypt.*

[b] *Botany & Microbiology Department, Faculty of Science, Al-Azhar University (girls branch*

Abstract

Individuals with specific metabolic disorders, such as phenylketonuria, require a diet low in phenylalanine for the duration of their lives. In this work, phenylalanine (Phe) was extracted by absorption from whey protein, casein, and skim milk powder enzymatic hydrolysates using activated carbon, barium sulfate, or a combination of activated carbon and barium sulfate in order to prepare dietary foods for patients with phenylketonuria. Initially, the amount of phenylalanine for different dairy proteins was determined by HPLC. Three hydrolysates' enzymes were prepared, using a protease+papain, pepsin or papain and the percentages of phenylalanine removal after enzyme hydrolysates followed by absorption treatments were detected by HPLC. The results indicated different phenylalanine amounts 20.49, 5.50 and 41.36 mg/mL for the skim milk, whey protein and casein, respectively. The best treatment showed to the use of barium sulphate, which produced 100% of Phe removal from whey proteins using each tested enzyme. Also, the use barium sulphate was removal 99.56, 99.30 and 98.96% of Phe from casein and 99.56, 99.6 and 99.6% from skim milk when applied protease+papain, pepsin or papain, respectively for hydrolsates. Moreover, the usage activated carbon was the best for absorption Phe hydrolysates from different proteins. Also, when using combined activated carbon and barium sulphate, the removal percent was improved, but for whey protein with pepsin was dropped the percentage to 46.72%. In general, the using papain enzyme for different dairy proteins more effective or the same effect for hydrolase phe than using combined protease+papain.

Keywords: Phenylketonuria; Phenylalanine; Whey protein, Casein; Protease; Papain; Activated carbon; Barium sulphate.

1. Introduction

Inborn errors of metabolism are a group of hereditary diseases that includes hyperphenylalaninemia as a subtype. PKU, or phenylketonuria, is the most prevalent form of hyperphenylalaninemia.[1] Because hepatic phenylalanine hydroxylase, which converts phenylalanine (Phe) to tyrosine, is lacking, it is characterized by high blood levels of Phe and excessive excretion of its metabolites.[2] When infants lack phenylalanine hydroxylase, a specific treatment formula requires a distinct milk fraction. Patients with PKU have trouble hydrolyzing Phe to tyrosine under normal conditions because tyrosine is an essential amino acid in their diet. [3]

Untreated PKU typically results in irreparable brain impairment, necessitating expensive financial and social institutionalization. High Phe is neurotoxic, resulting in severe intellectual disability and other neurologic symptoms, such as seizures, tremors, ataxia, and autistic behavior. [4] Most patients with PKU who require lifelong care are found during the newborn screening. [5] Throughout childhood, maintaining proper Phe levels is essential, particularly for mental and behavioral processes. [6] In PKU, growth is one of the primary outcomes that must be taken into account, and its optimization is crucial. [7] The opinions of patients should be taken into account while creating and approving novel PKU medications and treatments. [8] Generally, Blood Phe levels are used to categorize PKU patients as either mild PKU (blood Phe: 600–1200 lmol/l), classic PKU (blood Phe: >1200 lmol/l), or non-PKU hyperphenylalaninemia (blood Phe: 120–599 lmol/l). [9] Over 50% of those impacted exhibit one of the less severe clinical manifestations. [9]

Consuming meat, fish, dairy products, nuts, beans, and other foods high in protein is strictly forbidden on the PKU diet. [10] Special low-protein (low Phe) products are allowed, as are measured portions of fruits and vegetables. [11] Although a supplemental formula is necessary to supply the remaining essential amino acids, many patients find it unpleasant due to its strong taste and odor.[12] Consequently, adherence is a significant medical issue and PKU management is taxing for patients and their families.[7] In order to lower the consumption of Phe, PKU diet management involves limiting the amount of natural protein in the diet and adding specific medical formulae to ensure adequate energy, vitamins, minerals, and necessary amino acids. [13] The primary goal of PKU treatment is to closely regulate the blood Phe level, primarily in the early stages of development. To enhance PKU patients' quality of life, innovative treatment approaches are required. [14]

*Corresponding author e-mail: manar_loco99@yahoo.com ; (Manar M. Abd El-Rahman).

Receive Date: 26 January 2025, Revise Date: 21 March 2025, Accept Date: 07 April 2025

DOI: 10.21608/ejchem.2025.355719.11216

PKU is common in people all over the world; however, it is more common in the Middle East than in the West. [15] The two types of protein substitutes that are commercially available for the treatment of PKU are monomeric diets, which are made up of a balanced mixture of free synthetic amino acids and totally devoid of Phe, and oligomeric diets, which are made up of protein hydrolysates that are Phe-free or have a reduced content. [16] The best possible sources for creating protein hydrolysates for patient nutrition are skimmed milk, casein and whey proteins because of their sufficient amino acid content, widespread commercial availability, and affordable price. [3] They are proteins high in Phe (2.4–9.0% by weight), nevertheless, thus post-hydrolysis processes are required to remove aromatic amino acids before they can be used in PKU patients' diets. [17] Prior research has mostly focused on skim milk products and milk proteins to extract Phe from these products using activated carbon in conjunction with enzymatic hydrolysis by hydrolysates derived from other microorganisms. **Lopes et al.** [18] employed enzymatic hydrolysates and activated charcoal to extract Phe from skim milk powder. Also, **Silvestre et al.** [19] altered the temperature, enzyme-substrate ratio, and enzyme type to create low Phe milk hydrolysates. A protease from *Aspergillus oryzae*, either alone or in combination with papain, was used in a study by **Silva et al**.[20] to remove Phe from skim milk powder using activated carbon and enzymatic hydrolysates. [20]

In this study, we outline the best enzyme for creating an enzymatic hydrolysate formula of casein, whey protein, and skim milk powder with low Phe levels that can be used in PKU patients' diets. Also, the data used activated carbon, barium sulphate or the combination for these agents to absorb the phenylalanine hydrolysate and detected the best one via HPLC analysis.

2. Materials and Methods

2.1. Materials

The dry skim milk, whey protein and casein were purchased from local market and reconstituted by dissolving them in demineralized water. Also, the three enzymes were used in the enzymatic proteolytic process; the 1st was Papain enzyme (Sigma chemical Co, USA), the 2nd was Pepsin enzyme (Sigma chemical Co. USA) and the 3rd was protease from *Aspergillus oryzae* (Sigma chemical Co. USA). The Adsorption materials as barium sulphate powder and activated carbon powder are used for adsorption of phenylalanine from the hydrolysate were purchased from Sigma chemical Co. USA.

2.2. Methods

2.2.1. Determination of phenylalanine content of proteins types (milk protein, whey protein and casein) using High-performance Liquid Chromatography (HPLC)

The concentration of phenylalanine in different proteins was determined using an HPLC system (Waters, USA). We weighed 0.5 g of each protein source separately and mixed with 8 mL HCL acid (6 M). All samples were hydrolyzed at 110 °C for 22–24h then placed in an incubator. [21] The supernatant was centrifuged at 5000 rpm for 10 min. 20 mL of each sample were injected on RPC18 column (3.9 9 150 mm) for 26 min. Mobile phase speed was 0.8 mL/min under 107–109 kgf/cm2 pressure. In the mobile phase, a mixture of acetonitrile (2:98) and phosphate buffer (pH = 3.5) was used, and UV detection at 214 nm at 25–30 °C.[22]

2.2.2. Proteins (milk protein, whey protein and casein) hydrolyze by different enzymes (protease, papain, pepsin).

Three hydrolysates for each protein source were prepared using pepsin (PE) or papain (PA), separated or using papain in association with a protease of *Aspergillus oryzae* (AO). The skim milk solutions at 0.35 g/100 mL (w/v), corresponding to a protein concentration of 0.125 g/100 mL, were prepared in 0.01 mol/L phosphate buffer, pH = 6.0, but the buffer was 0.01 mol/L HCl-KCl, pH = 1.9, when we use PE only. At first, all solutions were heated in a water-bath, at 80 °C for 10 min. Then, the temperature was raised until adjusted to 50 °C. Then, the enzymes PA, PE and AO were added in such a concentration to attain the desired enzyme: substrate ratio (**Table 1**). The hydrolytic reactions were stopped by lowering the temperature to 10 °C in an ice bath (H1, H2 and H3), increasing the pH to 8.0 with 12.5 mol/L NaOH solution (H4, H5 and H6) or reducing the pH to 3.0 with formic acid PA (min. 88%) (H7, H8 and H9). The hydrolysates were, finally, freeze dried (Labconco freeze dryer, 77500 model, Kansas City, MI, USA). For all hydrolysates, the total time of hydrolysis was 5 h.[23]

Table 1: Hydrolytic conditions employed for preparing different hydrolysates

Hydrolysates	Hydrolysis time (h)	E:S (g/100g)		
		AO	PA	PE
H1	AO (1h) + PA(4h)	10	20	-
H2	AO (1h) + PA(4h)	10	20	-
H3	AO (1h) + PA(4h)	10	20	-
H4	PE (5h)	-	-	1
H5	PE (5h)	-	-	1
H6	PE (5h)	-	-	1
H7	PA (5h)	-	1	-
H8	PA (5h)	-	1	-
H9	PA (5h)	-	1	-

H1, H4 and H7 for whey protein; H2, H5 and H8 for casein; H3, H6 and H9 for skim milk; E:S: enzyme: substrate ratio; PA: Papain; PE: Pepsin; AO: protease from *Aspergillus oryzae*; Temperature: 50 °C.

2.2.3. Lowering phenylalanine release from proteins hydrolysis by active carbon with/ or barium sulphate

2.2.3.1. Use activated carbon (AC) according to Hassler. [24]

The activated carbon (AC) was used with concentration 90 g/g of hydrolysed protein. We put the activated carbon inside a syringe (20 mL) which contain wool glass and a filter of nylon. Then, the hydrolysate solution (80 mg/10 mL) allowed to pass through the column and the elute was collected at 25 °C.

2.2.3.2. Use barium sulphate according to Helbig et al. [25]

We put the barium sulphate inside a syringe (20 mL) which contain wool glass and a filter of nylon. Then, the hydrolysate solution (80 mg/10 mL) allowed to pass through the column and the elute was collected at 25 °C.

2.2.3.3. Use the combination of activated carbon (AC) and barium sulphate

We put activated carbon (AC) and barium sulphate inside a syringe (20 mL) which contain wool glass and a filter of nylon. Then, the hydrolysate solution (80 mg/10 mL) allowed to pass through the column and the elute was collected at 25 °C.

2.2.4. Detection of phenylalanine contents for hydrolysed proteins using HPLC

The concentration of phenylalanine in different proteins after adsorption on activated carbon and barium sulphate was determined using an HPLC system (Waters, USA). We weighed 0.5 g of each protein source separately and mixed with 8 mL HCl acid (6 M). All samples were hydrolyzed at 110 °C for 22–24h then placed in an incubator**. [21]** The supernatant was centrifuged at 5000 rpm for 10 min. 20 mL of each sample were injected on RPC18 column (3.9 9 150 mm) for 26 min. Mobile phase speed was 0.8 mL/min under 107–109 kgf/cm2 pressure. In the mobile phase, a mixture of acetonitrile (2:98) and phosphate buffer (pH = 3.5) was used, and UV detection at 214 nm at 25–30 °C**.[22]**

2.2.5. Evaluating the efficiency of phenylalanine removal

According to Eq. (1), The efficiency of phenylalanine (Phe) which removed was calculated, where initial amount of Phe = amount of Phe which present in different protein sources, and final amount of Phe = amount of Phe which present in hydrolysates after adsorption on activated carbon and barium sulphate

$$\text{Phe Removal (\%)} = \frac{\text{initial amount of Phe} - \text{final amount of Phe}}{\text{initial amount of Phe}} \text{X } 100 \qquad (1)$$

2.2.6. Statistical analysis

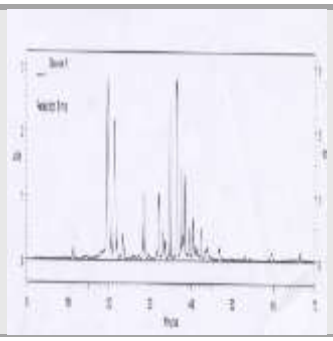
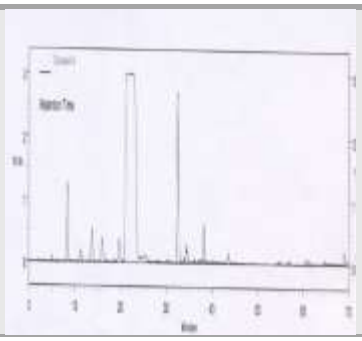
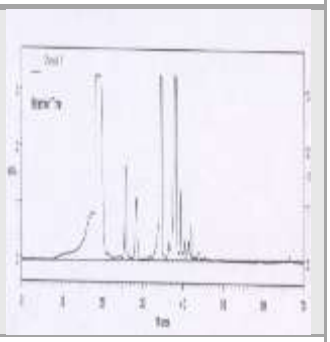
In this study all tests were performed in triplicate. (ANOVA) method was used for analysis of variance and Duncan test was used for comparison**. [26]**

3. Results and Discussion

3.1. Detection of phenylalanine content in different proteins.

Table (2) indicates the phenylalanine concentration that measured by HPLC. From the obtained results, the different sources of proteins were contained phenylalanine with concentration 20.49, 5.50 and 41.36 mg/mL for the skim milk, whey protein and casein, respectively. This amino acid was found with high amount of phenylalanine in whey proteins than others, which related to that whey proteins produced during cheese manufacturing and contained more types of amino acids related to the rennet enzyme effect and starter cultures strains more that casein and skim milk. These results were confirmed by **Silvestre et al.[27]** that indicated whey protein was fortified with high phenylalanine amounts and could remove it by protease enzyme. Also, other studies by **Lopez-Bajonero et al.[28]; Reinmuth-Selzle et al. [29]** found that different powder of milk as whey proteins and skim milk in general rich with amino acids included phenylalanine.

Table 2: Detection the phenylalanine content in different proteins using HPLC.

Protein sources	**Skim milk**	**Whey protein**	**Casein**
HPLC Graph			
Phenylalanine content (mg\mL)	20.49 ^{b}± 0.018	5.50 ^{c} ± 0.019	41.36 ^{a} ± 0.017

Each value represents the mean of triple determination. Different letters are significantly different ($p \leq 0.05$).

3.2. Effect of enzymatic action on phenylalanine removal

In this study different dairy protein (whey protein, casein and skim milk powder) as sources for phenylalanine were hydrolased via enzymes that may be used together (protease+papain) or in separate manner (pepsin or papain). The action of these enzymes was detected using HPLC after removal the hydrolsates phenylalanine via absorption using activated carbon, barium sulphate or combination the two agents together. These obtained results were displayed in the following Tables (**3, 4 and 5**) to determine the best hydrolased enzymes with perfect absorption agent. So, this formula be considered effective solution can be used for produce functional food products from milk to especial people who suffering from phenylketonuria.

3.2.1. Use of activated carbon to remove phenylalanine

Table (3) shows the affected of use activated carbon for phenylalanine adsorption after hydrolased with different enzyme for different protein types. So, the results from the enzymatic actions on different protein sources found that:

In case of whey protein (C1, C4 and C7), the using protease+papain (C1) and followed by activated carbon was removed the phenylalanine percentage about 83.45%. Also, the same effect was observed when used pepsin (C4), which the percentage of removal reached 83.81%. Moreover, the effect of papain (C7) was reached 84.36% of phenylalanine removal. In addition, the data did not indicate significant difference ($p \leq 0.05$) for the amount of phenylalanine removed using either protease+papain together (C1) or pepsin and papain in alone manner (C4 and C7).

In the case of casein (C2, C5 and C8), the usage activated carbon after casein hydrolyzed with different enzymes was detected reduction percent as; the use of protease+papain (C2) was giving reduction percent about 91.32%. In addition, the usage of pepsin (C5) for casein hydrolase was gave reduction percent about 91.32%. Also, the same percent of reduction was observed when used papain (91.24%). Therefore, no significant differences were indicated when used any tested enzymes applied on casein to remove phenylalanine with activated carbon.

Moreover, in the case of use different enzyme on skim milk (C3, C6 and C9) was indicated as percentages 69.79, 69.93 and 84.36 % when used protease+papain, pepsin and papain, respectively. Also, the data found that the high removal percent was reported by using papain with skim milk after applied activated carbon.

In general, the results demonstrated that no significant difference in the amount of phenylalanine removed was found in the case of casein and skim milk using either protease+papain together or pepsin and papain in alone manner. All in all, the high removal phenylalanine percent was observed for casein by using each tested enzymes, which the percent of removal after applied activated carbon was above 91.0%.

The results were confirmed by **Lopes et al.**[18] who used activated carbon to remove phenylalanine that hydrolysate from skim milk powder. The activated carbon was able to remove up to 99% of phenylalanine from skim milk hydrolysates, using protease and papain. Also, **Silvestre et al.**[27] found that the proteases from both *A. oryzae* and *Bacillus subtilis* produced the highest phenylalanine removals (79.0 and 77.8%, respectively).

Table 3: Efficiency of phenylalanine removal from different hydrolysates by activated carbon.

Hydrolysates	Removal of Phe (%)	Final Phe content (mg\mL Hydrolysates)
C1	$83.45^{b} \pm 1.2$	0.91 ± 4.7
C2	$91.32^{a} \pm 0.3$	3.59 ± 1.2
C3	$69.79^{c} \pm 0.1$	6.19 ± 0.5
C4	$83.81^{b} \pm 2.1$	0.89 ± 8.4
C5	$91.32^{a} \pm 0.6$	3.59 ± 2.5
C6	$69.93^{c} \pm 1.3$	6.16 ± 5.3
C7	$84.36^{b} \pm 0.2$	0.86 ± 0.8
C8	$91.24^{a} \pm 1.1$	3.62 ± 4.5
C9	$69.93^{c} \pm 1.3$	6.16 ± 5.3

Final Phe content = Concentration of phenylalanine after adsorption on activated carbon. Each value represents the mean of triple determination. Different letters are significantly different ($p \leq 0.05$).

3.2.2. Use of barium sulphate to remove phenylalanine

Table (4) demonstrates the use of barium sulphate for the adsorption of phenylalanine that results from the enzymatic hydrolysis on different protein sources found that:

In case of whey protein (B1, B4 and B7), the using protease+papain (B1) and followed by barium sulphate was removed all phenylalanine percentage (100.0%). Also, the same effect was observed when used pepsin (B4) and papain enzymes, which all tested enzymes able to remove all phenylalanine after applied with barium sulphate to absorb hydrolysed amino acid**.** So, no significant difference was observed for the amount of phenylalanine removed using papain and protease+papain as in B1 hydrolysate (100%) or pepsin and papain alone manner as in (B4 and B7) respectively.

In case of casein (B2, B5 and B8); the percentage of phenylalanine removal was reached to 99.56, 99.30 and 98.96 % when applied the enzymes protease+papain, pepsin and papain, respectively after applied barium sulphate as absorption agent. Additionally, the data did not indicate significant difference for the amount of phenylalanine removed by using either protease+papain (B2) (99.56%) or pepsin alone (B5) (99.3%). So, the usage one enzyme pepsin was more effective for phenylalanine removed and economic instated of used two enzymes as protease+papain. Also, the used hydrolysed pepsin enzyme (B5) was more efficient than the use of papain (B8).

The same trend of results was observed in skim milk case (B3, B6 and B9), which when used enzymes as protease+papain, pepsin and papain to hydrolyse phenylalanine and followed with absorption agent barium sulphat, the removal percent were recorded 99.56, 99.6 and 99.6%, respectively. So, no significant difference was observed for the amount of phenylalanine removed using either protease+papain together (B3) or pepsin and papain in alone manner (B6 and B9). The study by **Shehata et al.**[23] indicated that after adsorption to barium sulfate, the amount of free phenylalanine in the skim milk dropped from 6.34% to 0%, while after adsorption to activated carbon, it was 3.41%. According to **Silvestre et al.,**[19] who examined the impact of temperature and enzyme on phenylalanine removed, the maximum absorption of phenylalanine was demonstrated by protease at 50º C by barium sulphat.

Table 4: Efficiency of phenylalanine removal from different hydrolysates by barium sulphate.

Hydrolysates	Removal of Phe (%)	Final Phe content (mg\mL Hydrolysates)
B1	$100.0^{a} \pm 0.1$	0.00 ± 0.5
B2	$99.56^{a} \pm 0.3$	0.45 ± 1.2
B3	$99.56^{a} \pm 1.1$	0.09 ± 4.5
B4	$100.0^{a} \pm 0.1$	0.00 ± 0.5
B5	$99.3^{a} \pm 0.6$	0.29 ± 2.5
B6	$99.65^{a} \pm 0.2$	0.07 ± 0.8
B7	$100.0^{a} \pm 0.1$	0.00 ± 0.5
B8	$98.96^{b} \pm 1.2$	0.43 ± 4.7
B9	$99.6^{a} \pm 0.65$	0.08 ± 2.5

Final Phe content = Concentration of phenylalanine after adsorption on barium sulphate. Each value represents the mean of triple determination. Different letters are significantly different ($p \leq 0.05$)

3.2.3. Use of combination of activated carbon and barium sulphate to remove phenylalanine

The data in **Table (5)** clarifies the effect of combined activated carbon with barium sulphate on adsorption phenylalanine after hydrolysate with different enzymes. As the data, the usage different enzymes affected on whey protein (W1, W4 and W7) were found the removal percentage was recorded 90.0, 46.72 and 83.81 for protease+papain, pepsin and papain, respectively. The results were indicated the combined activated carbon with barium sulphate did not effective for absorb hydrolysate as using barium sulphate alone. These results could be related to antagonistic effect when used companied absorption agents. The un-accepted data found using combined activated carbon with barium sulphate on whey protein after hydrolases with pepsin was dropped the percentage of removal to 46.72 %. Also, a significant difference was observed in the amount of phenylalanine removed by using protease+papain together more than by using pepsin or papain alone.

Moreover, the effect of combined activated carbon with barium sulphate after hydrolaste casein (W2, W5 and W8) with different enzymes was recorded 98.67, 91.97 and 96.68 for for protease+papain, pepsin and papain, respectively. Also, the drop in the removal percent was observed for the effect of pepsin on casein (W5).

In addition, the same trend of results was observed for the skim milk (W3, W6 and W9), which the removal percent recorded 96.38, 81.05 and 92.28 % for protease+papain, pepsin and papain, respectively.

Table 5: Efficiency of phenylalanine removal from different hydrolysates by activated carbon and barium sulphate

Hydrolysates	Removal of Phe (%)	Final Phe content (mg\mL Hydrolysates)
W1	$90.0^{b} \pm 0.3$	0.55 ± 1.2
W2	$98.67^{a} \pm 1.2$	0.55 ± 4.8
W3	$96.38^{a} \pm 0.1$	0.47 ± 0.4
W4	$46.72^{d} \pm 2.2$	2.93 ± 8.8
W5	$91.97^{b} \pm 0.6$	3.32 ± 2.5
W6	$81.05^{c} \pm 2.1$	3.55 ± 8.4
W7	$83.81^{c} \pm 2.01$	0.98 ± 8.04
W8	$96.68^{a} \pm 0.2$	1.37 ± 0.8
W9	$92.28^{b} \pm 0.4$	1.58 ± 1.7

Final Phe content = Concentration of phenylalanine after adsorption on activated carbon and barium sulphate. Each value represents the mean of triple determination. Different letters are significantly different ($p \leq 0.05$).

4. Conclusion

This study was demonstrated the effect of different enzymes (Protease+papaein, pepsin or papain) on hydrolase dairy proteins sources as (whey protein, casein or skim milk) to reduce the Phe content after absorbate this hydrolase amino acid with activated carbon, barium sulphate or combined them together. The data designated to produce the suitable enzyme

and suitable absorption agent to can produce excellent dairy products for PKU patients. The important results were indicated that barium sulphate alone was more effective on absorb hydrolase Phe than using combined activated carbon with barium sulphate. Also, the activated carbon alone was effective too. Moreover, the applied papain enzyme for different dairy protein sources more effective or the same effect for hydrolase Phe than using combined protease+papain. So, the usage one enzyme was economic and gave the same effective target as combined enzymes. Additionally, it was possible and economic to applied whey protein (that waste produce from milk ultrafiltration during sot cheese production) to produce healthy products low in Phe content after treated this protein source with papain or other enzyme followed by using barium sulphate as absorption agent.

References

1. Alsharhan, H., & Ficicioglu, C. (2020). Disorders of phenylalanine and tyrosine metabolism. *Translational Science of Rare Diseases*, *5*(1-2), 3-58.
2. Burgard, P., Lachmann, R. H., & Walter, J. H. (2022). Hyperphenylalaninaemia. In *Inborn Metabolic Diseases: Diagnosis and Treatment* (pp. 337-354). Berlin, Heidelberg: Springer Berlin Heidelberg.
3. Soltanizadeh, N., & Mirmoghtadaie, L. (2014). Strategies used in production of phenylalanine-free foods for PKU management. *Comprehensive Reviews in Food Science and Food Safety*, *13*(3), 287-299.
4. Seddigh, A., Azadi, B., Ebrahimi, M., Ghafarizadeh, A., Koochmeshgi, J., & Hosseini-Mazinani, S. M. (2001). " Institute of Inherited Metabolic Disorders, General Faculty Hospitaland Ist Medical Faculty of Charles University. Prague, Czech Republic:"PKU Centre Dept of Paediatrics, University Hospital. *J. Inherit. Metab. Dis*, *24*, 1.
5. Berry, S. A., Brown, C., Grant, M., Greene, C. L., Jurecki, E., Koch, J., ... & Cederbaum, S. (2013). Newborn screening 50 years later: access issues faced by adults with PKU. *GeNetics in mediciNe*, *15*(8), 591-599.
6. Vockley, J., Andersson, H. C., Antshel, K. M., Braverman, N. E., Burton, B. K., Frazier, D. M., ... & Berry, S. A. (2014). Phenylalanine hydroxylase deficiency: diagnosis and management guideline. *Genetics in medicine*, *16*(2), 188-200.
7. Gama, M. I., Pinto, A., Daly, A., Rocha, J. C., & MacDonald, A. (2022). The impact of the quality of nutrition and lifestyle in the reproductive years of women with PKU on the long-term health of their children. *Nutrients*, *14*(5), 1021.
8. Hagedorn, T. S., van Berkel, P., Hammerschmidt, G., Lhotáková, M., & Saludes, R. P. (2013). Requirements for a minimum standard of care for phenylketonuria: the patients' perspective. *Orphanet journal of rare diseases*, *8*(1), 191.
9. Bartus, A., Palasti, F., Juhasz, E., Kiss, E., Simonova, E., Sumanszki, C., & Reismann, P. (2018). The influence of blood phenylalanine levels on neurocognitive function in adult PKU patients. *Metabolic Brain Disease*, *33*, 1609-1615.
10. Esra Gül1 & Ayşe Güneş Bayır (2024). Disease Management in Individuals with Phenylketonuria. Clin Exp Health Sci 2024; 14: 572-581 ISSN:2459-1459
11. Wood, G., Evans, S., Pointon-Bell, K., Rocha, J. C., & MacDonald, A. (2020). Special low protein foods in the UK: An examination of their macronutrient composition in comparison to regular foods. *Nutrients*, *12*(6), 1893. Gül, E., & Bayır, A. G. (2024). Disease Management in Individuals with Phenylketonuria. *Clinical and Experimental Health Sciences*, *14*(2), 572-581.
12. Daly, A., Evans, S., Pinto, A., Ashmore, C., & MacDonald, A. (2021). Protein substitutes in PKU; their historical evolution. *Nutrients*, *13*(2), 484.
13. Singh, R. H., Cunningham, A. C., Mofidi, S., Douglas, T. D., Frazier, D. M., Hook, D. G., ... & Rohr, F. (2016). Updated, web-based nutrition management guideline for PKU: an evidence and consensus based approach. *Molecular genetics and metabolism*, *118*(2), 72-83.
14. Delbreil, P., Dhondt, S., Kenaan El Rahbani, R. M., Banquy, X., Mitchell, J. J., & Brambilla, D. (2024). Current Advances and Material Innovations in the Search for Novel Treatments of Phenylketonuria. *Advanced Healthcare Materials*, *13*(26), 2401353.
15. Hillert, A., Anikster, Y., Belanger-Quintana, A., Burlina, A., Burton, B. K., Carducci, C., ... & Blau, N. (2020). The genetic landscape and epidemiology of phenylketonuria. *The American Journal of Human Genetics*, *107*(2), 234-250.
16. Levy, H. L., Sarkissian, C. N., & Scriver, C. R. (2018). Phenylalanine ammonia lyase (PAL): From discovery to enzyme substitution therapy for phenylketonuria. *Molecular Genetics and Metabolism*, *124*(4), 223-229.
17. Lara, M. G., Izumi, C., Greene, L. J., Vilela, L., & Freitas, O. D. (2005). Preparation and scaling up of a low phenylalanine enzymatic hydrolysate of bovine whey proteins. *Revista Brasileira de Ciências Farmacêuticas*, *41*, 459-466.
18. Lopes, D. C. F., Delvivo, F. M., & Silvestre, M. P. C. (2005). Use of activated carbon for removing phenylalanine from reconstituted skim milk powder hydrolysates. *LWT-Food Science and technology*, *38*(5), 447-453.
19. Silvestre, M. P. C., Souza, M. D., Junior, C. O. L., Silva, V. D. M., Aguiar, M. J. B., Afonso, W. O., & Silva, M. R. (2012). Effect of enzyme type, enzyme substrate ratio and Temperature on phenylalanine removal from milk. *American Journal of Food Technology*. 7(3), 123-132.
20. Silva, V. D. M., Marco, L. D., Afonso, W. O., Lopes, D. C. F., Januário, J. N., Aguiar, M. J. B., ... & Silvestre, M. P. C. (2007). Preparation of low-phenylalanine whey hydrolysates, using papain and pancreatin immobilized on activated carbon and alumina.

21. Fountoulakis, M., & Lahm, H. V. (1998). Hydrolysis and amino acid composition analysis of proteins. J Chromatogr A 826(2):109–134.
22. Piecyk M, Srama A, Bzducha AA (2007). Application of HPLC and GC/MS to quantification of phenylalanine in chosen kinds of food for particular nutritional use. Acta Sci Pol Technol Aliment 3058 J Food Sci Technol (July 2016) 53(7):3053–3059 123 6(2):5–8. http://www.food.actapol.net/issue2/volume/1_2_2007. Pdf
23. Shehata, A. E., El-Magdoub, M. N., Kamal, T. M., & Mohamed, H. A. (2008). enzymatic Preparation of low-Phenylalanine Formula Derived from Skim Milk Hydrolysate for Phenyl ketonuric Patients. *Egyptian Journal of Medical Human Genetics*, *9*(1), 51-71.
24. Hassler JW. Purification with activated carbon. 1st ed. New York: Chemical Publishing; 1974.
25. Helbig NB, Ho L, Christy GE, Nakai S. De Bittering of skim milk hydrolysates by adsorption for incorporation into acidic beverages. J. Food Sci. 1980; 45 (2): 331-5.
26. Pimentel-Gomes, F. (2000). "Curso de estatística experimental" 14. ed., Nobel, Piracicaba, 477 p
27. Silvestre, M. P., daSilva, M. C., deSouza, M. W., Silva, V. D., deAguiar, M. J., & Silva, M. R. (2013). Hydrolysis degree, peptide profile and phenylalanine removal from whey protein concentrate hydrolysates obtained by various proteases. *International Journal of Food Science and Technology*, *48*(3), 588-595.
28. Lopez-Bajonero, L. J., Lara-Calderon, P., Galvez-Mariscal, A., Velazques-Arellano, A., & Lopez-Munguia, A. (1991). Enzymatic production of a low-phenylalanine product from skim milk powder and casemate. *Journal of Food Science*, *56*(4), 938-942.
29. Reinmuth-Selzle, K., Tchipilov, T., Backes, A. T., Tscheuschner, G., Tang, K., Ziegler, K., ... & Weller, M. G. (2022). Determination of the protein content of complex samples by aromatic amino acid analysis, liquid chromatography-UV absorbance, and colorimetry. *Analytical and Bioanalytical Chemistry*, *414*(15), 4457-4470.