

Preparation of Enzymatic Skim Milk Hydrolysates with Low Phenylalanine Content

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SUMMARY. Papain (PA) and pepsin (PE) were used for preparing enzymatic hydrolysates from skim milk, in isolated mode and also in association with a protease from *Aspergillus oryzae* (AO). With the aim of using these hydrolysates for preparing dietary supplement for phenylketonurics, the activated carbon (AC) was then used to remove phenylalanine (Phe). Two types of treatment with AC were tested: stirring in a beaker and the use of a syringe as a column. In each case, some parameters were investigated. The best result was achieved using a 20 mL syringe and 90 g of hydrated carbon/g of casein. The isolated action of PA and PE, and the association of PA with AO produced the highest Phe removal (97% to 98%). The enzyme:substrate ratio studied here had no effect on the Phe removal.

RESUMEN. "Preparación de hidrolizados enzimáticos de leche desnatada con bajo contenido de fenilalanina". Papaína (PA) y pepsina (PE) fueron utilizadas en la preparación de hidrolizados enzimáticos de la leche desnatada, solas o en asociación con una proteasa de *Aspergillus oryzae* (AO). Con el objetivo de emplear estos hidrolizados para preparar un suplemente dietético para fenilcetonúricos, fue utilizado carbón activado (CA) para eliminar la fenilalanina (Phe). Dos tratamientos distintos con CA fueron ensayados: agitación en vaso de vidrio y uso de una jeringa como columna. En cada caso fueron evaluados algunos parámetros. El mejor resultado fue alcanzado al emplear una jeringa de 20 mL y 90 g del carbón hidratado/g de caseína. La acción aislada de la PA y de la PE, y la asociación de la PA con la AO produjeron la remoción más grande de Phe (de 97% hasta 98%). La relación enzima:substrato estudiada no tuvo ningún efecto sobre la remoción de la Phe.

INTRODUCTION

Protein hydrolysates have many applications in foods as in formulations planned to avoid allergies due to high molecular compounds since the decrease of chain length has direct relationship with the immunogenicity¹. Therefore they are used in special foods such as those destined to premature newborn, and to children presenting diarrhoea, gastroenteritis, malabsorption syndrome and phenilketonuria (PKU)^{2,3}.

The necessity of removing phenylalanine (Phe) from protein hydrolysates is associated to their use in the preparation of dietetic supple-

ments for PKU. In fact, the nutritional therapy for PKU is based on limitation of protein ingestion, reducing Phe supply to the minimum and promoting the normal growth of patients with other nutrients⁴⁻⁶.

Among several protein sources that may be used for preparing dietary supplements for phenylketonurics, isolated casein, the main milk protein, is the choice in most cases^{4,6,7}. However, in underdeveloped countries, this protein needs to be imported which represents an important increase in production costs. Thus, the use of alternative sources such as skim milk,

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containing proteins of high biological value, must be investigated. In Brazil, skim milk represents a by-product of milk industry with low utilization, contributing therefore to increase the pollution of the environment.

Considering that in our country the formulations normally used as dietetic supplements must be imported and, consequently, are high-price products, our interest turned to the preparation of these formulations, having protein hydrolysates as the main source of amino acids in a high available form, that is, in oligopeptide form, especially di- and tripeptides. This is the reason we have been preparing several protein hydrolysates and testing different hydrolytic conditions for obtaining peptide profiles appropriate for nutritional purposes⁸⁻¹⁴.

Most of the methods used for Phe removal from protein hydrolysates are based on the principle that a sufficient amount of Phe is liberated by enzymatic hydrolysis, and the free Phe is, then, removed by gel filtration, adsorption by activated carbon or resins^{4,6,7}.

In order to evaluate the efficiency of Phe removal, its amount must be determined either in the protein source or in their hydrolysates, after having used an appropriate adsorption method. Several techniques to quantify Phe among other amino acids are available in the literature, including the ion-exchange chromatography using the amino acid analyzer¹⁵, reverse high performance liquid chromatography¹⁶⁻²², hydrophilic interaction chromatography^{22,23} and spectrophotometric methods of second order (SDS)²⁴⁻²⁷.

Several authors reported the great reliability of using SDS, between 250 nm and 270 nm, for quantifying Phe in proteins, since parameters such as pH and the addition of other elements are controlled^{24,25,27-31}.

In a first study developed by our group¹³ using skim milk as protein source to prepare low-Phe products, we used a protease from *Aspergillus oryzae*, isolated or in several associations with papain, for preparing the hydrolysates, and we tested some parameters for removing Phe from these hydrolysates by stirring in a beaker.

The aim of the present study was the same as the first work, *i.e.*, to prepare skim milk hydrolysates containing low Phe content as protein source for producing dietary supplements for phenylketonurics. However, in this case, three enzymes were used (papain, pepsin and a

protease of *Aspergillus Oryzae*) for hydrolyzing the proteins and two procedures for removing Phe using activated carbon were compared (stirring in a beaker and the use of a column). The second derivative spectrophotometry was used for estimating the efficiency of the treatments in removing Phe from the hydrolysates.

MATERIAL AND METHODS

The L-phenylalanine (Phe), L-tyrosine (Tyr), L-tryptophan (Trp) and a protease from *Aspergillus oryzae* (XXIII type), pepsin and activated carbon were purchased from Sigma Chemical Co. (St.Louis, MO, EUA). Papain was kindly furnished by BIOBRÁS (Montes Claros, MG, Brazil). The skim milk was purchased in a supermarket of Belo Horizonte, MG, Brazil.

Preparation of skim milk hydrolysates

Five hydrolysates (H1, H2, H3, H4 and H5) were prepared using papain (PA) and pepsin (PE), isolated or 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, except when using only PE, where the buffer was 0.01 mol/L HCl-KCl, pH 1.9. Initially, the solutions were pre-heated in a water-bath, at 80 °C for 10 min. Then, the temperature was adjusted to 50 °C, and the enzymes PA, PE and a protease of AO were added in such a concentration to attain the desired enzyme:substrate ratio (Table 1). The hydrolytic reactions were stopped by reducing the pH to 3.0 with formic acid PA (min. 88%) (H1), increasing the pH to 8.0 with 12.5 mol/L NaOH solution (H2) or lowering the temperature to 10 °C in an ice bath (H3, H4 e H5). 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.

Preparation of standard curve of phenylalanine

Two standard curves were prepared, *i.e.*, one in 0.01 mol/L phosphate buffer, pH 6.0 for hydrolysates H1, H3, H4 and H5, and the other in 0.01 mol/L HCl-KCl buffer, pH 1.9 for hydrolysate H2. In both cases, the same procedure described bellow was followed for preparing the solutions and scanning the spectra.

Initially, stock solutions of Phe (6.05×10^{-4} mol/L), Tyr (5.52×10^{-4} mol/L) and Trp (4.90×10^{-4} mol/L)

Hydrolysates	Hydrolysis time (h)	E:S (g/100g)		
		AO	PA	PE
H1	PA (5h)	-	1	-
H2	PE (5h)	-	-	1
H3	AO (1h) + PE (4h)	1	-	2
H4	AO (1h) + PE (4h)	10	-	20
H5	AO (1h) + PA (4h)	10	20	-

Table 1. Hydrolytic conditions employed for preparing skim milk hydrolysates. E:S : enzyme:substrate ratio; AO: protease from *Aspergillus oryzae*; PA: Papain; PE: Pepsin; Temperature: 50 °C.

10⁻⁴ mol/L) were prepared in the same buffer solutions cited above. Then, 10 mL of each solution were mixed and successive dilutions of this mixture were made to have Phe concentrations in a range from 0.13 to 1.01 x 10⁻⁴ mol/L. Spectra of these diluted solutions were recorded from 250 to 280 nm (CECIL spectrophotometer, CE2041 model, Buck Scientific, England). A software GRAMS-UV (Galactic Industries Corporation, Salem, NH, EUA) was used to draw the second derivative spectra. For the standard curve, the area and height of 3^o and 4^o negative peaks of Phe spectra were tested in function of its concentration.

Use of Activated Carbon to Remove phenylalanine

Two procedures were tested for removing Phe from skim milk hydrolysates by the activated carbon (AC): stirring in a beaker and the use of a column (disposable syringe). The sample used in these tests was the hydrolysate H5.

Stirring in a beaker

In this case, the procedure described before by our group¹³ was used. In total, five quantities of AC were tested. Besides the amount we had the highest removal of Phe in this previous work (118 g/g of casein), four other quantities were tested here: 15, 30, 60 e 90 g/g of casein. Concisely, the AC was added to 80 mg/10 mL hydrolysate solutions in purified water (Aries, Vaponics, USA). The mixtures were stirred at 25°C in a velocity just to keep the carbon in suspension (Fisatom stirrer, São Paulo, SP, Brazil), centrifuged at 10,000 rpm for 10 min at 25 °C (Jouan centrifuge, BR4i model, France), and filtered (qualitative filter paper, Whatman n. 1, Maidstone, England).

Using a column

The AC (varied quantities) was placed inside a disposable syringe of 20 mL containing a filter of nylon and wool glass, manufactured in our

laboratory. The hydrolysate solution (80 mg/10 mL) was added to the column and the eluate was collected at 25 °C. Four tests were made, as described bellow.

Test 1. Type of contact between the hydrolysate and the AC. Three procedures were tested: a) Direct flow: the hydrolysate was passed directly through the AC; b) Wait contact: the hydrolysate was held in contact with the AC for 30 min inside the column before elution was started and c) Dripping: the hydrolysate was let fall in drops on the AC.

Test 2. Hydration of activated carbon. The AC was previously stirred with 25 mL of purified water for different times (5, 10, 15, 20 and 30 min), before being placed in the syringe.

Test 3. Capacity of the syringe. Two kinds of syringes were tested: 10 mL and 20 mL, using the hydrated AC and the direct flow procedure.

Test 4. Amount of activated carbon. Three quantities of hydrated AC were tested: 60, 90 and 118 g/g of casein.

Quantification of phenylalanine

Initially, the samples of skim milk and its hydrolysates were hydrolysed (5.7 mol/L HCl, 110 °C, 24 h). Then, their absorbance was measured from 250 to 280 nm, as described for the standard curve. Second derivative spectra were drawn and the areas or heights of negative peaks were used to calculate the amount of Phe in the samples, employing the standard curve. In case of protein hydrolysates, this same procedure was employed after the treatment with AC.

Evaluating the Efficiency of Phe Removal

The efficiency of Phe removal was calculated according to Eq. (1), where initial amount of Phe = amount of Phe in skim milk, and final amount of Phe = amount of Phe in hydrolysates treated by activated carbon.

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

Statistical Analysis

All experiments were carried out in triplicate. Differences between means of areas or heights were evaluated by analysis of variance (ANOVA) and Duncan test³². Differences were considered to be significant at $p < 0.05$ throughout this study. The least square method was used to fit the standard curve and the adequacy of the linear model ($y = ax + b$) was tested at $p < 0.05$. The factorial analysis was used to evaluate Phe removal comparing the two treatments (beaker and column), in different concentrations of activated carbon. The analysis of variance was performed for each condition, in order to investigate the presence of significant effects among treatments ($p < 0.05$), and in these cases the Duncan test was applied to establish the differences among the means³².

RESULTS AND DISCUSSION

Standard curve of phenylalanine

The equation for the curve prepared in 0.01 mol/L sodium phosphate buffer (pH 6.0) was $y = 2.1748x + 0.3913$ and $R^2 = 0.992$; while that for the curve prepared in 0.01 mol/L HCl-KCl buffer was $y = 6.0452x - 0.2375$ and $R^2 = 0.972$. In both curves, the area of negative peak "d" (Figure 1) was chosen since it showed the high-

est coefficient of determination (R^2). Also, the regression for Phe, in pH 6.0 and 1.9, was highly significant ($p < 0.001$). These results are in agreement with others in the literature^{33,34} and also with previous studies carried out in our laboratory^{11,13,35}, since in all these works linearity for the standard curve of Phe, in presence of Tyr and Trp in several concentrations, was shown.

On the other hand, in some reports in the literature, the number of negative peaks for Phe was different from that found in the present work. Thus, five peaks for this amino acid in pH 7.0 were shown by some authors³³, while others³⁶ described the presence of six, in pH 1.9. These discrepancies could be associated to several factors such as forms of Phe (free or ester), type of equipment (spectrophotometer and software used for measuring absorption and derivative spectra), properties of the solvent and pH used^{26,33,37}.

SDS Spectra of phenylalanine

The SDS spectra of Phe, in pH 6.0 and 1.9, as well as those of hydrolysates H1 (pH 6.0) and H2 (pH 1.9) are shown in Figure 1 (c-d). A similarity among the spectra of hydrolysates in both pH values and between hydrolysates and

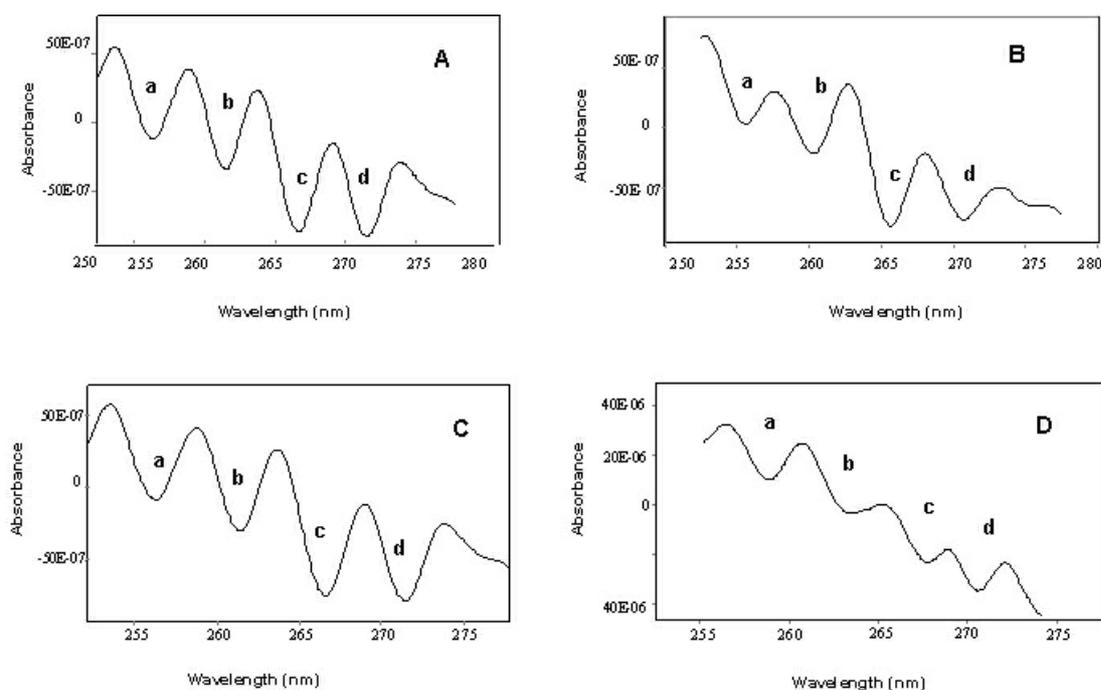


Figure 1. Second derivative spectra of Phe in pH 6.0 (A) and pH 1.9 (B) in a solution containing Tyr and Trp and of hydrolysates H1 in pH 6.0 (C) and H2 in pH 1.9 (D).

standard Phe can be observed, considering the number and position of negative peaks.

The likeness among the spectra of standard amino acids and proteins had previously been described by some authors ³³, working with several native and denatured proteins. Also, no difference between the SDS spectra of protein hydrolysates and standard Phe was observed in our laboratory using PA for hydrolysing casein ¹¹ and a protease from AO for hydrolysing skim milk ¹³.

**Sitirring in a beaker:
selection of the amount of activated carbon**

As shown in Table 2, the amount of AC placed in a beaker influenced the Phe removal. Among the five quantities of AC tested, the use of 90 g and 118g/g of casein gave similar results which were superior of that using 60 g/g of casein.

**Using the column:
selection of the best conditions**

No significant difference was observed between two of the procedures tested for the contact between the hydrolysate and the AC, *i.e.*, direct flow and wait contact, which removed 97% and 98% of Phe, respectively. Contrarily, the amount of Phe removed by the dripping procedure (95%) was significantly smaller than the two first ones. It is worth stating that among these three procedures, the direct flow shows the advantage of being the simplest and fastest one.

Considering the hydration of the AC, the agitation time of 10 min was necessary to produce an appropriate adsorption of water. Also, the use of the hydrated AC in a direct flow by the column slightly increased the Phe removal of 1%, in comparison with the wet AC.

The capacity of the syringe influenced significantly the Phe removal which was of 97% and 93% for the 20 mL and 10 mL syringes, respectively.

Contrarily to the results observed for the treatment in a beaker, the data in Table 2 show that the amount of AC used in the column had no influence in the Phe removal, since no significant difference was observed among the values found.

Treatment in a beaker versus column

In this case, the data used for the column correspond the best results found in the direct flow, hydration of AC for 10 min and syringe of 20 mL.

According to the results in Table 2, the only difference between these two treatments refers to the use of 60 g of AC/g of casein, where the use of a column was slightly superior of that of a beaker. Moreover, the treatment in a column eliminates three stages of the Phe removing technique (stirring, centrifugation and filtration), which reduces either the time or the costs of the process.

Although no difference was observed in the level of Phe removal using different AC amounts, 90 g of AC/g of casein was chosen for the treatment of skim milk samples due to its higher feasibility of spreading on the filter inside the column, compared to 60 g of AC/g of casein.

Amount of phenylalanine removed

The use of AC in the conditions established in previous item was efficient in removing Phe from all skim milk hydrolysates (Table 3), having reached levels changing from 94% to 98%, which correspond to final Phe amount of 25.2 and 7.4 mg of Phe / 100 g of hydrolysate, respectively. The Phe quantity found in the skim milk was of 2,048 mg/100 g.

No data was found in the literature concerning the Phe removal from skim milk hydrolysates. Some authors ⁴ reported a smaller Phe removal (92%) from casein hydrolysates than that of the present work. This discrepancy may be related to the higher amount of activat-

Treatments		Amount of activated carbon (g/g casein)		
		60	90	118
Beaker	%	95.4 ^{2/b} ± 2.1	97.3 ^{1/a} ± 0.3	98.2 ^{1/a} ± 0.6
Column*	%	97.3 ^{1/a} ± 0.4	98.1 ^{1/a} ± 0.6	96.6 ^{1/a} ± 1.2

Table 2. Effect of the amount of activated carbon concentration on the phenylalanine removal. *Direct flow, hydrated carbon; syringe of 20 mL. Sample = hydrolysate H5. Each value represents the mean of triple determination. Different letters are significantly different (p ≤ 0.05) for different treatments of the same carbon concentration. Different numbers are significantly different (p ≤ 0.05) for the same treatment at different carbon concentrations.

ed carbon used here (38.5 times). Nevertheless, no mention was made by these authors regarding the conditions of activated carbon use, such as the length and the velocity of stirring, as well as the temperature of the protein solution, which could also interfere in the Phe removal. Moreover, although these authors stated that they used skim milk and casein as substrate, the results presented in their paper involved only casein.

Although we used a larger amount of activated carbon than some authors ⁴, the advantage of this work is related to the lower hydrolysis length (5 times) and the use of only one enzyme, which are important factors to reduce costs and increase purity of these formulations. It is well known that using a reaction time longer than 5 h to hydrolyse proteins, increases the probability of microbial contamination ^{6,38-40}.

In other works, the activated carbon was used to remove Phe from protein hydrolysates. Thus, some authors ⁴¹, after hydrolysing whey proteins with actinase, in pH 6.5 at 37 °C, treated these preparations with activated carbon and removed 97% of Phe. However, the conditions for the treatment with activated carbon were not mentioned. Other authors ⁴⁰ removed 95% of Phe from casein hydrolysates. However, these authors employed more severe conditions than those used here, *i.e.*, a very long time for hydrolysis (72 h) and for the treatment with activated carbon (5.5 h).

Some authors employed other methods, in order to remove Phe from protein hydrolysates, such as gel filtration ⁶. However, the removal efficiency was not mentioned by these authors. The use of polystyrene resins (XAD-4 e XAD-16) was reported previously ⁷, leading to an almost complete removal of Phe (99.9%). However, this method has the disadvantage related to the much higher cost of these resins compared to activated carbon ^{4,6,40}.

Effect of enzymatic action over the phenylalanine removal

Isolated action of the enzymes

No difference was observed for the amount of Phe removed using either PA or PE in isolated mode, as shown in Table 3 for hydrolysates H1 and H2, respectively.

The effect of the isolated action of enzymes was also studied by other authors, who reported similar results for the Phe removal in comparison to the present work (over 90%). In these works different protein sources were tested and

Hydrolysates	Removal of Phe* (%)	Final Phe content (mg/100 g Hidrolysate)
H1	97.6 ^a ± 1.2	9.6 ± 4.7
H2	97.1 ^a ± 0.3	11.5 ± 1.2
H3	94.7 ^b ± 0.1	20.9 ± 0.5
H4	93.6 ^b ± 2.1	25.2 ± 8.4
H5	98.1 ^a ± 0.6	7.4 ± 2.5

Table 3. Efficiency of Phe removal from skim milk hydrolysates by activated carbon. * Use of a column (direct flow), 90 g of hydrated carbon/g of casein, syringe of 20 mL. Final Phe content = Phe content after treatment with activated carbon. Each value represents the mean of triple determination. Different letters are significantly different ($p \leq 0.05$).

the activated carbon or adsorption resins were used for removing this amino acid from protein hydrolysates ^{7,41,42}.

Association of enzymes

As shown in Table 3, among the three associations studied, those that employed PE with AO (hydrolysates H3 and H4) gave similar results and were less efficient than the association of PA with AO (hydrolysate H5), in removing Phe.

Some authors ⁴², used pronase E associated with PA or chymotrysin and reported similar amounts of Phe removed (98,8%) to those of the found here for the association of PA with AO. These authors also employed pronase associated with PE and their results for Phe removal were superior (99.8%) to the association of PE with AO in the present work. In other studies ⁴, the association of AO with PA was also used and removed less Phe than here (92%,) although they used a much longer reaction time (20 h). Using a system of three enzymes containing chymotrypsin, carboxypeptidase A and leucine aminopeptidase A report in the literature ⁴⁰ indicated a removal of 95% of Phe from casein hydrolysates, inferior to the levels found in our study.

The association of PA and PE used by some authors ⁴³ for preparing casein hydrolysates with low Phe content after treatment with activated carbon was inefficient since it removed only 36% of this amino acid.

Isolated action versus association of enzymes

Comparing the isolated action of PA (H1) with its association with AO (H5), the data in Table 3 show that both conditions were similarly efficient in the removal of Phe. On the other hand, when PE acted alone (H2), it was more

advantageous than its association with AO (H3 and H4) leading to a higher Phe removal.

Contrarily to the present study, some reports found in the literature showed that the association of enzymes gave better results than their isolated action. That was the case of the use of pronase E alone and its association with PE (99.0% and 99.7% of Phe removal, respectively)⁴². Also, the association of AO with PA (E:S = 1% and 2%, respectively), showed to be more advantageous for removing Phe from casein hydrolysates than the use of these enzymes separately⁴.

Effect of E:S ratio

The comparison of the results for the hydrolysates H3 and H4 (Table 3) shows that the ten-fold increase in the E:S ratios of both enzymes (AO and PE) had no effect on the Phe removal. Contrarily, in a previous work of our group, this same increase of the E:S ratios of the two enzymes used in association (AO with PA)

to hydrolysis skim milk reduced the Phe removal from 99% to 96%¹³. No other data was found in the literature concerning the effect of the E:S ratio on the Phe removal.

CONCLUSION

The activated carbon (AC) removed 94% to 98% of Phe from enzymatic hydrolysates of skim milk. Among the different conditions tested for the treatment with AC, the use of a syringe of 20 mL as a column in a direct flow, with 90 g of AC/g of casein gave the best result. The isolated action of PA and PE produced similar level of Phe removal. No advantage was shown for the use of the association of these enzymes with AO in terms of Phe removal compared to their isolated action. Increasing the E:S of the enzymes was inefficient in improving Phe removal.

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