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BIOCATALYSIS

Reduction of L-phenylalanine in protein hydrolysates using L-phenylalanine ammonia-lyase from *Rhodosporidium toruloides*

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Abstract L-Phenylalanine ammonia-lyase (PAL, EC 4.3.1.25) from *Rhodosporidium toruloides* was utilized to remove L-phenylalanine (L-Phe) from different commercial protein hydrolysates. A casein acid hydrolysate (CAH, L-Phe ~2.28 %) was employed as a model substrate. *t*-Cinnamic acid resulting from deamination of L-Phe was extracted, analyzed at $\lambda = 290$ nm, and used for PAL activity determination. Optimum reaction conditions, optimized using successive Doehlert design, were 35 mg mL^{-1} of CAH and 800 mU mL^{-1} of PAL, while temperature and pH were 42 °C and 8.7, respectively. Reaction kinetics of PAL with CAH was determined under optimized conditions. Then, removal of L-Phe from CAH was tested. Results showed that more than 92 % of initial L-Phe was eliminated. Similar results were obtained with other protein hydrolysates. These findings demonstrate that PAL is a useful biocatalyst for L-Phe removal from protein hydrolysates, which can be evaluated as potential ingredients in foodstuffs for PKU patients.

This paper is dedicated to the memory of our dear colleague, Prof. Carlos F. Mignone, who recently passed away.

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Introduction

Phenylketonuria (PKU) is an inborn error of metabolism in which there is a persistent increase in serum L-Phe concentration due to a total or partial decrease in L-phenylalanine hydroxylase (PAH, EC. 1.14.16.1) activity [8]. PAH is the liver enzyme required for hydroxylation of L-Phe to L-tyrosine (L-Tyr).

Although PKU is one of the most common inborn metabolic diseases all over the world (Table 1), there is no consensus on the classification of PKU. In general, four categories are recognized: classic PKU (serum L-Phe levels greater than 1200 μmol L^{-1}), moderate PKU (600–1200 μmol L^{-1}), mild PKU (360–600 μmol L^{-1}), and mild hyperphenylalaninemias (m-HPA) (120–360 μmol L^{-1}) [8].

PAH deficiency is an autosomal-recessive disorder. The gene is located on the long arm of chromosome 12 where more than 400 pathological mutations were recognized [9]. Additionally, PAH needs (6*R*)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH_4) as cofactor, molecular oxygen and an active site-bound Fe^{2+} for the bioconversion of L-Phe to L-Tyr (Fig. 1) [17]. The deficiency in the biosynthesis or recycling of the BH_4 cofactor was described as one of the causes of a HPA subtype, which can be partially treated by BH_4 supplementation [19].

The purpose of PKU treatment is to maintain plasma L-Phe levels within a safety range (120–360 μmol L^{-1}), where no neurological damage is observed [8]. PKU patients are unable to metabolize L-Phe, resulting in an L-Phe increase in the blood stream thus affecting the

Table 1 PKU incidence worldwide (adapted from refs. [7, 29])

Country	PKU incidence	Country	PKU incidence
Argentina	1:28,200	Italy	1:17,000
Australia	1:10,000	Japan	1:125,000
Brazil	1:23,600	Korea	1:41,000
Canada	1:22,000	Mexico	1:161,700
Chile	1:19,600	Norway	1:14,500
China	1:17,000	Paraguay	1:31,600
Costa Rica	1:49,200	Peru	1:48,900
Cuba	1:52,600	Scotland	1:5300
Finland	<1:100,000	Spain	1:6500
France	1:13,500	Turkey	1:2600
Germany	1:8500	UK	1:14,300
Hungary	1:11,000	Uruguay	1:20,900
India	1:18,300	USA	1:15,000
Ireland	1:4500	Yemenite Jews (in Israel)	1:5300

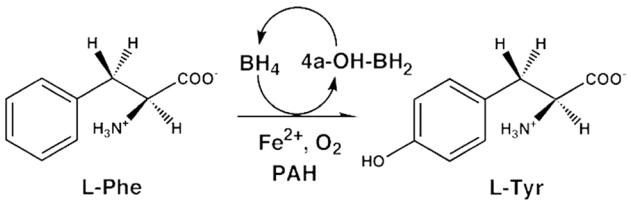

Fig. 1 Phenylalanine hydroxylase (PAH) reaction [17]

central nervous system. Whichever the case, and depending on severity, PKU patients tolerate only minimal amounts of L-Phe in their diet. Dietary therapy with restriction of Phe intake remains the mainstay of treatment for PAH deficiency, requiring a decrease in the consumption of natural protein and replacing it with a protein source (amino acid mixture) devoid of L-Phe [27]. Nowadays, several free-Phe formulas are available in the market. Some of them include a synthetic preparation of amino acids supplemented with vitamins and minerals. Moreover, in the search for natural protein diets, some alternative supplements were developed; one example is the glycomacropeptide (GMP), an intact whey protein naturally low not only in L-Phe but also in other amino acids (tyrosine, histidine, leucine, tryptophan, and arginine). GMP was extensively studied for its use as an alternative for amino acids formulas [21]. Finally, it should be mentioned the use of large neutral amino acids (LNAA) as a potential alternative for current amino acid preparations. LNAA compete with L-Phe as a blood brain barrier for entry to the brain thorough the same transporter (LAT1) [9], so the supplementation with LNNA decreases the brain Phe level providing substrates for the neurotransmitters and brain proteins synthesis. However, the strategies for LNAA supplementation for this objective remain in the study [26].

As it was exposed above, there are several alternatives for PKU diet. Nevertheless, some of them are not available worldwide [9] or they have a high cost, in particular for undeveloped countries [25]. In many developed countries, social security systems completely refund the cost of PKU treatment; however, in other countries (particularly undeveloped and developing countries), the cost of special low-protein or protein-free foods is not totally paid for the social security systems and the patient must be in charge of the treatment costs [13]. This fact suggests the need for novel treatments and/or alleviation procedures, especially for undeveloped and developing countries. Consequently, a less expensive amino acid supplementation for PKU patients, produced by a methodology easy to apply by any food additive industry, could be an interesting attempt to provide alternative diets to patients all over the world.

L-Phenylalanine ammonia-lyase (PAL, EC 4.3.1.25) catalyzes the conversion of L-Phe to *t*-cinnamic acid (*t*-CA) and ammonia [18]. PAL was first described in higher plants in relation to defense mechanisms [18]. Later, PAL expression was detected in certain strains of *Rhodotorula* sp. grown on L-Phe as a sole carbon and nitrogen source [22]. The highest PAL activity was found in *Rhodotorula glutinis* NBRC 0559 (later renamed as *Rhodosporidium toruloides*) grown in a medium containing L-Phe as inducer [23]. PAL has been extensively purified and characterized in a large number of plants [15, 28] and some microorganisms [1, 12, 14, 16]. In addition, PAL has been widely studied due to its potential use in relation to PKU. Many studies have reported the utilization of PAL in the PKU diagnostic test in infant's urine [5] as well as in PKU treatment [3, 4]. More recently, recombinant PAL was stabilized by pegylation with polyethylene glycol (PEG-PAL) and used for the depletion of L-Phe in the blood stream, mostly by subcutaneous route [20, 24]. These underway studies are very much promising for PKU treatment. However, at this moment, dietary therapy remains the mainstay of treatment. In this context, utilization of PAL to reduce L-Phe content in foodstuffs has not been reported yet. The present work aims to use PAL for L-Phe removal from some commercially available protein hydrolysates, in order to provide alternative dietary supplements for PKU patients.

Materials and methods

Materials

DEAE-Toyopearl was from Tosoh Co., Tokyo. L-Phe and L-isoleucine were from Wako Pure Chemical Co., Osaka.

Phenylmethanesulfonyl fluoride (PMSF) was from Sigma-Aldrich Co., St. Louis. The protein hydrolysates were purchased from the following companies: casein acid hydrolysate (CAH, Casamino acids) from Oxoid Co., Hampshire; acid casein peptone from Britania Co., Buenos Aires; Bacto peptone from Difco Co., Detroit; meat peptone from Sigma-Aldrich Co.; gelatin peptone from Merck Co., Darmstadt; and tryptone from Biokar Co, Beauvais. All other chemicals employed were commercially available and of analytical grade.

Microorganism and culture conditions

The *R. toruloides* NBRC 0559 strain, used for PAL production, was maintained in a medium containing 20 g L-Phe, 2.5 g K_2HPO_4, 1 g KH_2PO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.5 g yeast extract (Oxoid Co.), 20 g sucrose, and 15 g agar (per liter). The optimal culture medium reported by Yamada et al. [30] was used for PAL production. In this case, the culture medium consisted of 10 g yeast extract, 10 g Bacto peptone, 5 g NaCl, 0.5 g L-Phe, and 5 g L-isoleucine (pH = 6.0) (per liter). Yeast cultivation was performed aerobically at 30 °C and 200 rpm on a rotary shaker for 30 h. Cells were separated by centrifugation (4000 rpm, 5 min, 4 °C) and the biomass obtained was used as a PAL source.

Partial purification of PAL

PAL was partially purified following the protocol reported by Adachi et al. [1]. The first step included cell disruption by three successive passages through an X-press. The obtained cell suspension was centrifuged at 10,000 rpm for 20 min, and the supernatant was heated at 50 °C for 5 min, with constant stirring. Heat-aggregated precipitates were removed by centrifugation under the above conditions. Afterward, the supernatant was loaded onto a column of DEAE-Toyopearl and the enzyme was eluted with 30 mM KCl solution. The resulting enzyme solution, containing 2800 mU mL^{-1}, was lyophilized and kept at −20 °C until use.

Standard enzyme assay

PAL activity (under standard conditions) was measured by monitoring the increase of optical density at 290 nm (OD_{290}) caused by *t*-CA formation when assayed in a reaction mixture containing 10 μmol of L-Phe, 200 μmol of Tris–HCl buffer (0.1 M, pH = 8.5), and enzyme solution at 30 °C, according to Ogata et al. [23]. A unit of PAL activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of *t*-CA per minute.

Optimization of PAL reaction conditions with casein acid hydrolysate (CAH) as a model substrate

A partially purified PAL from *R. toruloides* was used to reduce L-Phe content in CAH (Oxoid L41, AN/TN = 64 %, L-Phe ~2.28 % w/w). A reaction mixture containing 5 g L^{-1} of CAH dissolved in Tris–HCl buffer (0.1 M, pH 8.0) and PAL at 140 mU per mL was incubated at 30 °C for 2 h and used for the preliminary assays.

Reaction conditions were optimized from calculating the maximum enzyme efficiency obtained within experimental ranges of substrate concentration, enzyme concentration, pH, and temperature. Reactants concentration and reaction conditions were optimized using response surface methodology (RSM), by Doehlert hexagonal designs [11]. This statistical matrix is generated with an equally spaced distribution of points on a concentric spherical shell, giving more uniformity than other experimental designs. First, a range of substrate (0–50 g L^{-1}) and enzyme concentrations (0–280 mU mL^{-1}) were tested by incubating the reaction mixture (pH 8) with PAL at 30 °C for 2 h. In parallel, for a fixed amount of substrate and enzyme, the reaction pH (7 to 9) and temperature (30–70 °C) were optimized. In both cases, the reaction was monitored by measuring the increased of the optical density at 290 nm.

In order to describe the response observed, a full quadratic model was used

$$Z = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i<j} \beta_{ij} X_i X_j,$$

where Z is the dependent response, X_i are the independent variables included in the model, k the number of effects, β_0 a constant, β_i the linear coefficient, β_{ii} the quadratic coefficient, and β_{ij} the interaction effect coefficient. In this study, Z represents the OD_{290}, $k = 2$, X_1 and X_2 are the variables in each RSM (CAH/PAL and T/pH), and the parameters β_0, β_1, β_2, β_{11}, β_{22}, and β_{12} are the regression coefficients calculated for each RSM using ANOVA.

Once the parameters were optimized, the reaction kinetics of PAL with CAH as substrate was determined under these conditions.

Enzyme assay in complex reaction mixtures

In order to measure PAL activity in the presence of a complex substrate (amino acid mixture), a novel method was developed. In this method, a solution of CAH in Tris–HCl buffer (0.1 M, pH 8.0) was treated with a partially purified PAL. The reaction was terminated at different reaction times by adding trichloroacetic acid solution (final concentration: 2 % w/v). Then, the sample was centrifuged

(10,000 rpm, 10 min) and the resulting *t*-CA (reaction product) was extracted from the supernatant with ethyl acetate in a 20:1 proportion (1 mL of ethyl acetate plus 50 μL of sample). Finally, the *t*-CA was measured spectrophotometrically in the organic phase at 290 nm (OD_{290}). For reaction optimization, incubation was performed during 2 h, while the reduction of L-Phe content was measured after overnight incubation.

Quantification of L-Phe by HPLC

L-Phe content in complex amino acid mixtures treated with PAL was evaluated by high performance liquid chromatography (HPLC) using a methodology adapted from Atherton and Green [6]. The injector used was Waters 717 plus Autosampler, with an injection volume of 20 μL per sample, at room temperature. The chromatographic separation was performed using a C_{18} reverse phase column (150 × 4.6 mm, 5-μm particle size, Waters). The mobile phase utilized was a solvent mixture of phosphoric acid (pH = 2):methanol (80:20) with an isocratic flow rate of 1 mL min^{-1}. Finally, a Waters 2996 photodiode array detector was operated at 257 nm. The standard curve of L-Phe (up to 500 μmol L^{-1}) was obtained using a 99 % purity standard (Biopack). Reaction mixtures were diluted accordingly in the solvent mixture.

Effect of protease inhibitors in PAL activity

In order to prevent proteolysis of PAL during the time course of the reaction, two kinds of protease inhibitors were tested: ethylenediaminetetraacetic acid disodium salt (EDTA-Na_2), a metalloprotease inhibitor, was used at the final concentration of 5 mM in the reaction mixture, and the other inhibitor used was phenylmethanesulfonyl fluoride (PMSF), a serine protease inhibitor, at a final concentration of 0.5 mM. Treatments with combination of both inhibitors and a control without inhibitors were carried out as well. All experiments were performed at optimal temperature in Tris–HCl buffer (0.1 M, pH 8.3). Reaction mixtures were incubated overnight, and L-Phe contents (initial and final) were analyzed by HPLC.

Use of PAL for the treatment of different protein hydrolysates

In order to determine if PAL is effective onto other complex substrates, 6 commercial products obtained from various protein raw materials treated with different hydrolysis procedures (chemical or enzymatic) were tested under optimal reaction conditions. The following substrates were used: yeast extract, acid casein peptone, Bacto peptone, meat peptone, gelatin peptone, and tryptone. All experiments were performed overnight at 42 °C in Tris–HCl buffer (0.1 M, pH 8.3), containing 5 mM of EDTA-Na_2. In each case, initial and final L-Phe contents were determined by HPLC as described above.

Results and discussion

Optimal reaction conditions of PAL with CAH

Previous studies [22] report the optimal reaction parameters for purified PAL using L-Phe as a sole substrate in a defined reaction mixture. In general, these parameters depend on the enzyme origin, i.e., microorganisms or plants. In this study, the optimal parameters were determined using a more complex substrate (CAH) containing a mixture of amino acids and small peptides in addition to other minor compounds (i.e., inorganic salts). Both the enzyme and substrate concentrations in reaction mixture were optimized in relation to PAL activity using a Doehlert experimental design. According to ANOVA, both parameters were statistically significant ($p < 0.05$) on PAL activity ($R^2 = 98.2$ %).

As shown in Fig. 2, the model predicted that OD_{290} increases along with substrate and enzyme concentrations and it does not apply to low substrate (<17 g L^{-1}) and enzyme (<105 mU mL^{-1}) concentrations. The highest PAL activity was predicted at

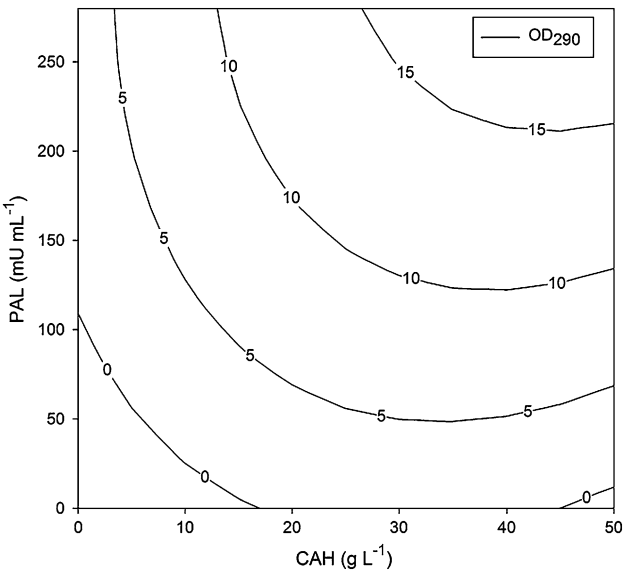

Fig. 2 Contour plot showing the effect of substrate and enzyme concentrations in PAL activity (determined by increment in OD_{290}). CAH (0–50 g L^{-1}) and enzyme concentrations (0–280 mU mL^{-1}) were tested by incubating the reaction mixture (pH 8) at 30 °C for 2 h. For the experimental design, a Doehlert hexagonal matrix was used and the results were described using a full quadratic model

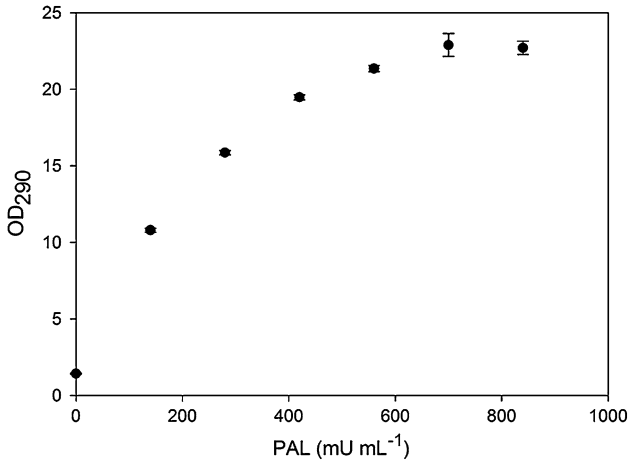

Fig. 3 Influence of PAL concentration in reaction mixture on PAL activity (determined by the increase of OD_{290}). For a fixed amount of CAH (35 g L^{-1}), successive increments of PAL were assayed in order to determine the saturating concentration of PAL. Reaction mixture (pH 8) was incubated at 30 °C during 2 h

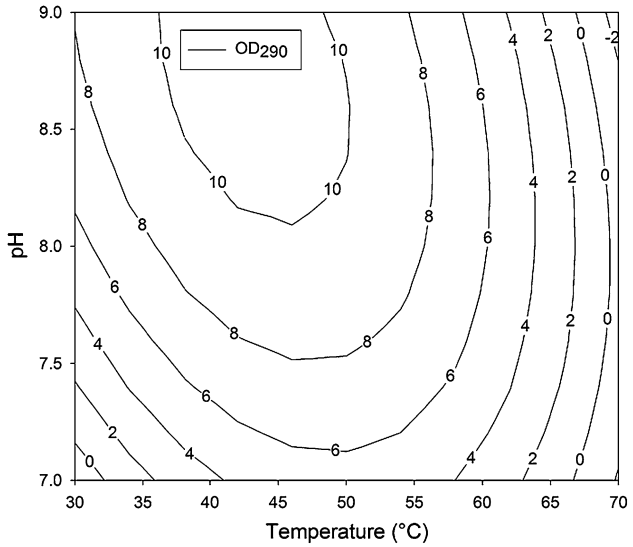

Fig. 4 Contour plot showing the effect of reaction conditions in PAL activity (determined by increase of OD_{290}). The reaction pH (7–9) and temperature (30–70 °C) were optimized by incubating 10 g L^{-1} of CAH with 140 mU mL^{-1} for 2 h. For the experimental design, a Doehlert hexagonal matrix was used and the results were described using a full quadratic model

CAH concentrations close to its maximum solubility (≅50 g L^{-1} w/v, previously determined) and at PAL concentrations ≅ 280 mU mL^{-1}. Consequently, the effect of enzyme concentrations higher than 280 mU mL^{-1} was tested with 35 g L^{-1} of CAH and gradual increments of PAL. In this case, OD_{290} increased to a maximum at ≅800 mU mL^{-1} of PAL (Fig. 3).

Table 2 Increment of PAL activity (determined by OD_{290}) after optimization of pH and temperature

Parameter	Initial condition	Optimized condition
Temperature (°C)	30	42
pH	8.0	8.7
OD_{290}	25.460	57.280

Substrate (CAH): 35 g L^{-1}, PAL: 800 mU mL^{-1} and reaction time: 2 h

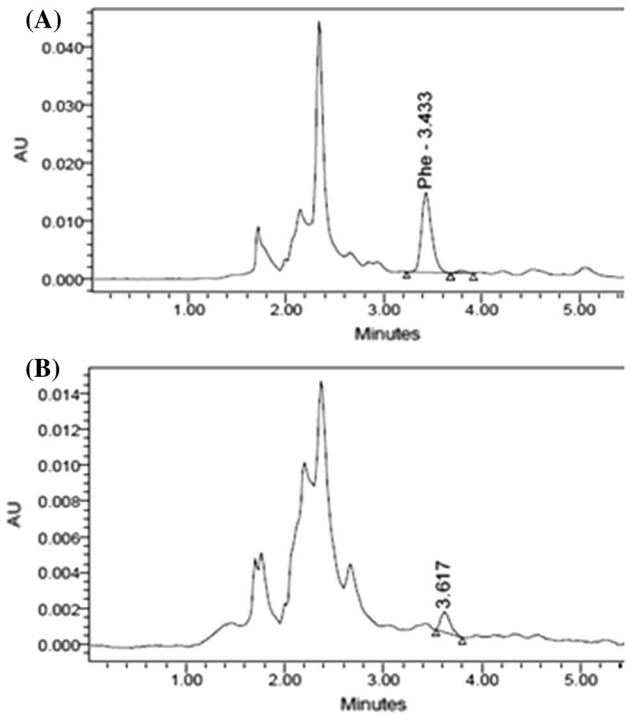

Fig. 5 HPLC chromatogram of casein acid hydrolysate (CAH) treated with PAL under optimal reaction conditions (35 g L^{-1} of CAH, 800 mU mL^{-1} of PAL, pH 8.3 and 42 °C). **a** Initial stage; **b** overnight reaction. The area coordinate of chromatogram B was magnified to visualize the peak

In addition, pH and temperature were also optimized using a uniform shell design. In this case, both parameters were statistically significant ($p < 0.05$) on PAL activity, with a negative effect of temperature and a positive effect of pH (R^2 = 98.0 %). As illustrated in Fig. 4, the maximum PAL activity was obtained within the following ranges: 37–50 °C for temperature and 8.2–9.0 for reaction pH. These findings showed a highly significant correlation with the studies of Ogata et al. [22] with L-Phe as substrate. Finally, the comparison of the optimized processing variables (pH and temperature) with the original reaction conditions indicated that PAL activity increased significantly (more than two times) (Table 2).

Table 3 Effect of protease inhibitors (EDTA and PMSF) in PAL reaction with CAH as substrate

Treatment	Initial L-Phe content (μmol L^{-1})	Final L-Phe content [μmol L^{-1} (reduction percentage)]
PSMF	4805	1164 (76)
EDTA	4917	376 (92)
PMSF + EDTA	4891	369 (92)
No inhibitor	4992	1134 (77)

CAH solution was prepared to a final concentration of 35 g L^{-1} in Tris buffer (0.1 M, pH = 8.3) and treated overnight at 42 °C with PAL (800 mU mL^{-1}). Results were obtained by HPLC determinations

Reduction of L-Phe content in CAH treated with PAL in the presence of protease inhibitors

Preliminary assays revealed an unexpected decrease in PAL activity during early reaction stages of PAL with CAH. The possibility of thermal inactivation of PAL was investigated, but it was soon discarded after obtaining the same kinetic pattern. As result, this observation was tentatively ascribed to the potential presence of proteases in the reaction mixture, probable traces of yeast intracellular proteases which remain after PAL partial purification process. Therefore, addition of two different protease inhibitors (PMSF and EDTA-Na_2 as well as their combination) to the reaction mixture was tested.

HPLC determination of L-Phe in standard samples resulted in a highly significant correlation between L-Phe concentration and peak area ($R^2 = 0.9998$) with a peak at a retention time of ≅3.6 min. HPLC chromatograms revealed the characteristic peak of L-Phe in samples taken at the initial stage of the CAH containing reaction mixture. The maximum decrease in L-Phe content was obtained under optimized reaction conditions in the presence of EDTA-Na_2 (Fig. 5). These results suggest that PAL could be affected by the presence of metalloproteases, after prolonged incubation. PAL was stabilized by the incorporation of a chelating agent like EDTA in the reaction mixture. In addition, EDTA has a bacteriostatic effect, avoiding substrate spoilage during incubation. Under these conditions, 92 % of L-Phe originally present in CAH was eliminated (Table 3). Conversely, PMSF did not show any significant effect when compared with the blank that contained no inhibitor. These results may vary depending on the nature of the substrate. Thus, the occurrence of residual proteases should be considered, particularly in those products obtained by enzymatic procedures (i.e., Bacto peptone, meat and gelatin peptones, and tryptone).

The determination of PAL kinetics with CAH as substrate under optimal reaction conditions in Tris–HCl buffer (0.1 M, pH 8.3, containing 5 mM of EDTA-Na_2) showed that production of *t*-CA (assayed at 290 nm) increased linearly with reaction time up to at least 6 h of incubation (Fig. 6). This fact suggests the positive effect of EDTA-Na_2 to avoid loss of PAL activity. Nevertheless, an enzyme preparation with stronger PAL activity is required in order to reduce the duration of enzyme treatment, in view of potential industrial applications.

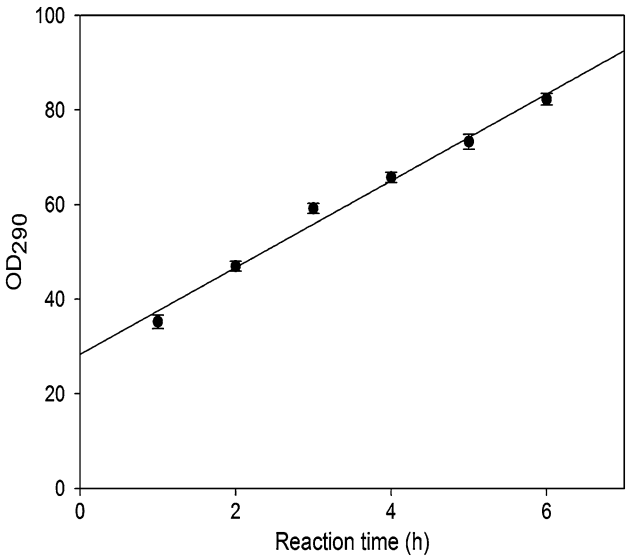

Fig. 6 Reaction kinetics of PAL under optimized conditions (35 g L^{-1} of CAH, 800 mU $mL^{-1}$of PAL, pH 8.3 and 42 °C) (OD_{290} = 9.1718 × time + 28.345, $R^2 = 0.987$)

It should be mentioned that protease inhibitors can be used only in laboratory experiments. The use of PMSF and EDTA (as well as any other preservatives and chemicals) should be avoided in the actual diet preparation for PKU patients. Although minimal, contribution of L-Phe content from residual PAL to the final product should be also considered. For practical industrial production, suitable countermeasures should be developed.

Use of PAL for the treatment of different protein hydrolysates

The results obtained with CAH encouraged the use of PAL with various commercial protein hydrolysates as alternative substrates. Yeast extract (obtained by yeast autolysis), acid casein peptone (prepared by acid hydrolysis), and

different enzymatic hydrolysates: Bacto peptone (enzymatic digest of animal tissues), meat (peptic digest) and gelatin (pancreatic digest) peptones, and tryptone (pancreatic digest of casein) were evaluated. In all treated substrates, a substantial decrease of L-Phe content, determined by HPLC, was recorded after an overnight treatment with PAL at 42 °C in the presence of EDTA as protease inhibitor. The reduction level (expressed as percentage of the original L-Phe content) was substantially high for the different substrates evaluated. As shown in Table 4, L-Phe could not be detected in final reaction mixtures originally containing yeast extract, gelatin peptone, and tryptone. In the other cases, L-Phe level obtained after PAL incubation with acid casein peptone, Bacto peptone, and meat peptone reached a minimum (Fig. 7). The differences observed could be ascribed to various factors, the residual protease activity from the manufacturing process among others. Nevertheless, in all cases, the remaining amount of L-Phe was lower than 900 μmol L^{-1}, which is equivalent to ≅4 mg L-Phe per g of protein (Phe/Pr). The recommended daily protein intake (contained in amino acid formulas) for classical PKU children is ~2–3 g kg^{-1} day^{-1} for infants <1-year old and ~1–2 g kg^{-1} day^{-1} for infants >1-year old [2]. Additionally, the minimal amount of Phe required for normal development of children with classical PKU is 20 mg kg^{-1} day^{-1} [10]. Taking into consideration these recommendations, we can conclude that the remaining concentration of L-Phe in all treated samples would result innocuous for PKU patients. In addition, residual *t*-CA is a harmless product that is

Table 4 Reduction of L-Phe content in various commercial protein hydrolysates treated with PAL

Substrate	Reduction (%)	Remaining L-Phe	
		(μmol L^{-1})	Phe/Pr (mg g^{-1})
Yeast extract	100	ND	0
Casein acid peptone	94	172	0.8
Bacto peptone	84	806	3.8
Meat peptone	81	852	4.0
Gelatin peptone	100	ND	0
Tryptone	100	ND	0

All substrates were prepared with a concentration of 35 g L^{-1} in Tris buffer (0.1 M, pH = 8.3) and treated with PAL with a final concentration of 800 mU mL^{-1}. Reactions were performed overnight at 42 °C and residual amounts of L-Phe were determined by HPLC. The ratio between the final Phe and protein content (Phe/Pr) was calculated and compared with the dietary recommendations for PKU patients

ND not detected

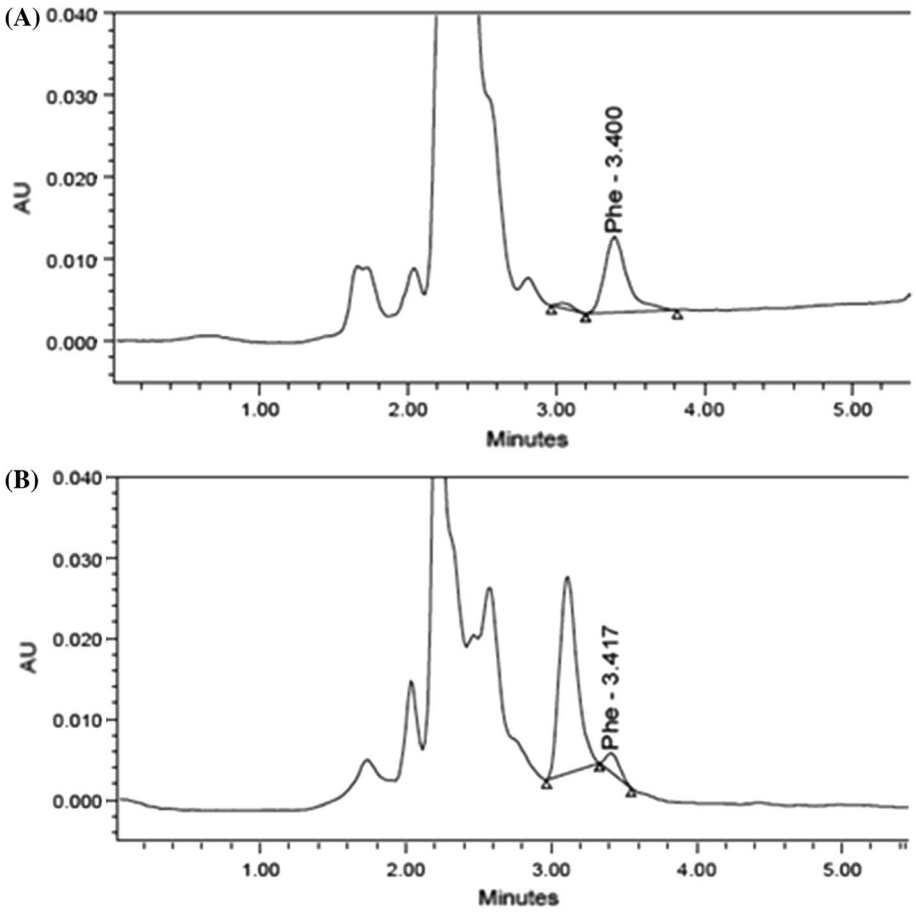

Fig. 7 HPLC chromatogram of Bacto peptone treated with PAL under optimal reaction conditions (35 g L^{-1} of CAH, 800 mU $mL^{-1}$of PAL, pH 8.3 and 42 °C). **a** Initial stage; **b** overnight reaction. The new peak that appears after L-Phe removal is an artifact formed during incubation and it has no critical meaning itself

degraded further to benzoic acid, which in turn is converted into rapidly excreted hippuric acid. The amount of produced ammonia does not pose a threat of hyperammonemia.

Finally, it should be mentioned that PAL from *R. toruloides* (EC 4.3.1.25) is also capable to catalyze the deamination of L-tyrosine (L-Tyr) to *p*-coumaric acid but at a substantially low reaction rate [1, 16]. Therefore, the final content of L-Tyr in samples treated with PAL should be analyzed in each case and taken in consideration to avoid its deficiency in PKU patients. Consequently, future studies must evaluate the need for supplementation of these protein hydrolysates with L-Tyr in each particular case. Alternatively, there is a specific PAL (EC 4.3.1.24) for L-Phe, which was isolated from barley seed by Koukol and Conn [18]. However, the purified plant PAL (EC 4.3.1.24) has a very low activity compared with microbial PAL (EC 4.3.1.25) obtained from *R. toruloides*. Therefore, microbial PAL (EC 4.3.1.25) could be more suitable for potential industrial applications.

Conclusions

PAL from *R. toruloides* is able to reduce the original L-Phe content in different commercial protein hydrolysates substantially, in some cases up to negligible values. This promising result may be considered a starting point for the production of mixtures of amino acids with low (or null) L-Phe content useful for the preparation of customized foods and/or dietary supplements for PKU patients. These mixtures may become a new generation of ingredients with low L-Phe content, highly competitive with amino acids mixtures currently available in the market. Thus, it seems reasonable to consider that the enzymatic elimination of L-Phe in a commercial protein hydrolysate will be more economical than the production of a mixture of different amino acids from the individual synthetic ones.

Studies of different alternatives for PAL immobilization as well as the scaling up of the process are in progress and the corresponding results will be eventually published.

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