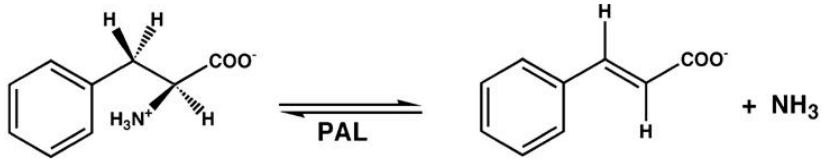
OPTIMIZATION OF REACTION CONDITIONS FOR PHENYLALANINE AMMONIA-LYASE WITH CASEIN HYDROLYSATE AS MODEL SUBSTRATE

Castañeda, M. Teresita[1]; Adachi, Osao[2]; Hours, Roque A[1].

[1]Center for Research and Development of Industrial Fermentations (CINDEFI; UNLP, CONICET La Plata) School of Science, La Plata National University. 47 y 115, (B1900ASH) La Plata, Argentina.
e-mail: castaneda@biotec.quimica.unlp.edu.ar
[2]Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515 Japan.

Introduction

L-Phenylalanine ammonia-lyase (PAL, EC 4.3.1.25) is a microbial enzyme that catalyzes the bioconversion of L-phenylalanine (L-Phe) to *t*-cinnamic acid (*t*-CA) and ammonia.



L-Phe ***trans*-cinnamic acid**

This enzyme was first described in higher plants in relation to defense mechanisms. Some years later, PAL was isolated from some genera of yeast. The highest PAL activity was shown by *Rhodotorula glutinis* NBRC 0559 (later renamed as *Rhodosporidium toruloides*) grown in a medium containing L-Phe as inducer. PAL has been extensively purified and characterized in a large number of plants and some microorganisms.
In recent years, PAL has been widely studied for its potential use in the treatment of phenylketonuria (PKU), a genetic disorder caused by deficiency of L-phenylalanine hydroxylase (PAH, EC. 1.14.16.1). PKU patients are unable to catalyze conversion of L-Phe to L-Tyr, resulting in an increment of L-Phe in blood stream affecting the central nervous system. If the PKU patient is not cared immediately with an special diet with low L-Phe content, several mental retardation will occur.
It is known from previous studies which parameters are optimal for purified PAL using L-Phe as a sole substrate. In general, these parameters depend on the origin of the enzyme, i.e.: microorganism or plants. In this study, the optimal parameters were determined for a complex substrate used as a model for the reduction of L-Phe content in protein hydrolysates.

Methodology

A partially purified PAL obtained from *R. toruloides* was used for the treatment of casein acid hydrolysate (CAH, OXOID L41, AN/TN = 64%, L-Phe ~ 2.28 % w/w), in order to reduce L-Phe content.
The reaction conditions were optimized in terms of: substrate (0-50 g/l) and enzyme concentrations (0 to 280 mU/ml), buffer pH (7 to 9) and temperature (30 to 70ºC), using successive response surfaces, by Doehlert hexagonal designs. In order to describe the response observed, a full quadratic model was used. The coefficients were calculated by ANOVA.

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

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

In order to measuring the activity of PAL with a complex substrate, a novel method was developed. In this method, a solution of CAH in Tris-HCl buffer (0.1 M, pH 8.0), with addition of 5 mM 2NaEDTA, was treated with partially purified PAL at 30°C. The reaction was terminated at different reaction times by addition of a volume equal to 10 % (v/v) of trichloroacetic acid (TCA, 20 % w/v). Then, the sample was centrifuged at 10,000 rpm for 10 min and the resulting *t*-CA (reaction product) was extracted from the supernatant with ethyl acetate in a proportion 20 : 1. Finally the *t*-CA was measured spectrophotometrically in the organic phase at 290 nm (OD_{290}). Due to the fact that PAL from *R. toruloides* is capable to use L-Tyr as well as L-Phe as substrates, it was needed to quantify the actual reduction of L-Phe content by HPLC.

Results

By Doehlert design, the enzyme and substrate concentrations were optimized in relation to PAL activity. According to ANOVA, both parameters were statistically significant ($p<0.05$) onto PAL activity (R^2 = 98.2 %). As it was expected, the optical density increased with an increment of substrate and enzyme concentrations, obtaining the highest activity of PAL using concentrations closed to the maximum solubility of CAH (50 g/l w/v). Enzyme concentration was tested with 35 g/l of CAH and successive increments of PAL. In this case, OD_{290} increased until 800 mU/ml of the enzyme and then OD_{290} leveled off.

On the other hand, pH and temperature were also optimized using uniform shell design. In this case, both parameters were statistically significant ($p<0.05$) onto PAL activity, with a negative effect of temperature and a positive effect of pH (R^2 = 98 %). The maximum PAL activity was achieved at temperatures between 40°C and 45°C and a reaction pH from 8.2 to 8.7.

Finally, by comparing the variables of processing (pH and temperature) with the standard reaction conditions, PAL activity was increased two times.

Under these conditions, production of *t*-CA assayed at 290 nm increased linearly at different times of treatment, reducing the L-Phe content, as was detected by HPLC measurements.

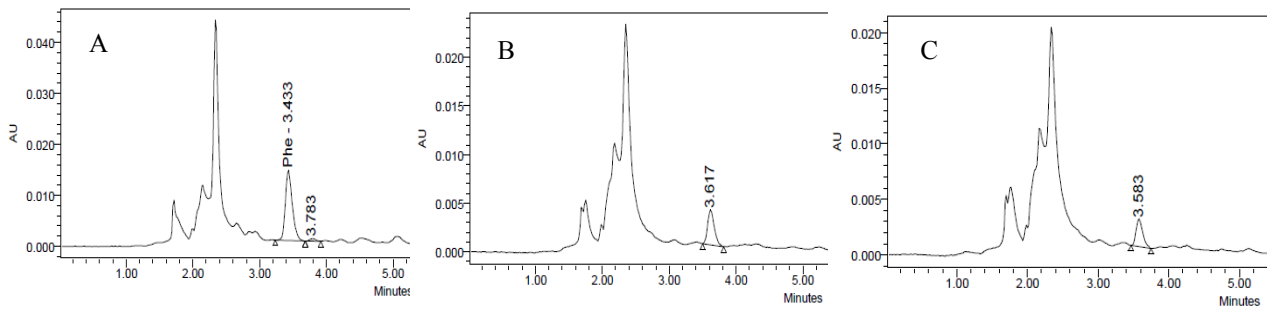


Figure: Chromatograms for the samples treated with PAL under optimal reaction conditions A) Original substrate; B) 3 h of treatment; C) 6 h of treatment. Note: the coordinates have been magnified to appreciate the picks.

Conclusion

In this study, optimization of reaction parameters was performed by using uniform shell design, increasing PAL activity two times in comparison with the original procedure. By using the optimized parameters, kinetics of PAL was linear at early reaction times, resulting in a substantial reduction in of L-Phe content in CAH, as it was confirmed by HPLC measurements. The product thus obtained can be considered as a potential ingredient for the preparation of foods for PKU patients.