

A Biochemical Method for Tyrosine Determination in Phenylketonuria Using a Colorimetric Enzymatic Approach

COSMIN STEFAN MOCANU¹, ANA-SIMONA BOCEC^{2,3}, VASILE ROBERT GRADINARU^{1*}, DANA-TEODORA ANTON-PADURARU^{2,3*}

¹Alexandru Ioan Cuza University of Iasi, Faculty of Chemistry, 11 Carol I Av., 700506, Iasi, Romania

²Grigore T. Popa University of Medicine and Pharmacy, Faculty of Medicine, Mother and Child Department, 16 Universităţii Str, 700115 Iasi, Romania

³St. Maria Children Emergency Hospital, 62 Vasile Lupu Str, 700309, Iasi, Romania

Abstract: Phenylketonuria is a serious genetic disease caused by a deficiency of phenylalanine metabolism, an essential amino acid found in daily nutrition. This disorder is caused by the lack of a specific enzyme called phenylalanine hydroxylase which mediates the conversion of phenylalanine to tyrosine). Thus, after ingestion of phenyl alanine-rich proteins, the amino acid concentration increases considerably in the blood due to proteolysis. Genetic defects of enzymes responsible for phenylalanine metabolic conversion were intensively studied. Among them the defect of gene encoding phenylalanine hydroxylase has a higher notoriety. This genetic defect is translated in an inactive enzyme constructs that impair the aminoacid hydroxylation. This physiological stage is also called hyperphenylalaninemia where slightly high levels of phenylalanine are noticed in the blood or urine. Consequently, the amino acid is converted to phenylpyruvic acid by transamination, the later displaying a particularly toxic effect against brain tissues. The aim of this study was to quantify tyrosine in the blood of patients suffering of phenylketonuria by an alternative enzymatic method. The tyrosinase used in this assay was extracted from commercial mushrooms (*Agaricus bisporus*) following the Haghbeen protocol with some modifications. Two chromatographic steps (molecular exclusion chromatography and ionic exchange chromatography) were used during the enzyme purification process. High purity samples were concentrated using ultrafiltration. The tyrosinase was screened by a classical enzymatic microplate assay having DOPA as a substrate. Finally the pure enzyme was used in order to quantify tyrosine from different standard solutions. The level of tyrosine from deproteinized serum samples was determined using a similar enzymatic strategy.

Keywords: phenylketonuria, tyrosinase, enzyme extraction, chromatography, spectroscopy

1. Introduction

Phenylketonuria (PKU) is an autosomal recessive inborn disorder characterized by a deficiency in L-phenylalanine metabolism. This disorder was firstly reported in 1934 by Abjorn Folling. Phenylketonuric patients have elevated phenylalanine (L-Phe or F) concentrations in their tissues due to lack or partial deficiency of phenylalanine hydroxylase (PAH). Therefore, patients have low tyrosine concentrations [1]. Phe is an essential amino acid derived exclusively from the diet or by endogenous proteolysis. This amino acid is crucial for protein biosynthesis and is a precursor for tyrosine (L-Tyr or Y) and its derivatives such as dopamine, adrenaline and melanin [2]. Decarboxylation and transamination of F are two minor alternative pathways that produce metabolites like phenylethylamine or phenylpyruvate [3]. First enzymatic reaction relies on L-phenylalanine conversion to phenylethylamine in catecholaminergic neurons. Phenylethylamine might potentiate neurotransmission by inducing synapsis depolarization (due to release of dopamine) [4]. However, monoamine oxidase B (MAO-B), an enzyme with low activity in PKU newborns, prevents phenylethylamine accumulation [5]. The phenylalanine transamination to phenylpyruvic acid (PPA) is a reaction assisted by phenylalanine transaminase (PAT) that usually occurs in liver. The majority of PPA is excreted in urine via renal system. In turn, the remained PPA is converted to phenyllactic acid (PLA). Two research studies support

*email: robert.gradinaru@uaic.ro, antondana66@yahoo.com



a correlation between serum phenylalanine and phenyllactic acid especially at elevated amino acid concentration. Moreover, brain PLA levels are considerable higher for phenylketonuria mouse model. These findings could be and indicator of potential acute neurotoxicity of PLA in PKU patients [6, 7].

The main metabolic Phe pathway relies on PAH enzymatic activity. In physiological conditions this tetrameric enzyme is expressed mainly in the liver [8]. PAH have the tetrahydrobiopterin (BH₄) as a cofactor and catalyzes the conversion of L-Phenylalanine to L-Tyrosine [9]. In turn, L-tyrosine is converted to L-dihydroxyphenylalanine (L-DOPA) and later to dopamine by L-DOPA decarboxylation. These two reactions are catalyzed by tyrosine hydroxylase (TH) and DOPA decarboxylase. Some studies reveal that L-DOPA oral medication may cause digestive dysfunctions in patients diagnosed with Parkinson's disease [10]. Both tyrosine and L-DOPA are involved in melanogenesis [11]. Dopamine is a neurotransmitter that binds to several brain receptors [12] and an intermediate that intervene in the physiopathology of atopic dermatitis [13]. Tyrosine is also essential for thyroid hormone synthesis. Thyroglobulin/thyroid peroxidase (TPO) system is used in thyroxine (T₄) synthesis [14]. T₄ regulates actin polymerization, and Ca²⁺-ATP-ase activity, in specific cells or tissues [15].

Since PAH assisted reaction is substrate dependent, its relevance should be considered in the events associated with high Phe blood levels [17]. Phenylalanine is competing with Tryptophan and Tyrosine for an amino acid transporter (blood-brain barrier). In consequence, serotonin, norepinephrine and dopamine brain levels are significantly decreased [18]. Phenylketonuric patients were distinguished by serious neurological damages including corpus callosum, striatum and cortical alterations. Some strategies as neutral amino acid supplementation proved to have clinical benefits on affected patients [19, 20]. Anyway, the pathophysiology underlying the brain damage due to F accumulation is still not completely understood. One of the hypotheses claims that F and its metabolites might act as neurotoxins in the brain by forming amyloid-like deposits [21]. Two enzymes, superoxide dismutase (SOD) and catalase (CAT), linked with oxidative stress, were affected by higher Phe concentration [22]. The bioenergetics impairment (mitochondrial metabolic disturbances) might also induce neurodegeneration [23]. The oligodendrocytes incapacity to maintain the myelin to required level decrease the axon ability to conduct action potential [24]. Occasionally, progressive white matter degeneration was observed in adult PKU patients. Also, a developmental arrest of cerebral cortex was reported so far in such patients [25].

The aim of this study was to quantify the concentration of Y from the blood of patients suffering of PKU by an enzymatic approach using tyrosinase. Immobilized phenylalanine ammonia lyase (PAL) from *Rhodotorula glutinis* has been used for phenylalanine quantification from urine [26]. Another attractive alternative could be represented by tyrosinase, an enzyme that converts the amino acid tyrosine in colored metabolites. Our attempt was to isolate a less expensive enzyme that is easier produced [27, 28] and it has potential clinical applications.

2. Materials and methods

2.1. Reagents

All solutions were prepared using ultrapure water from a Millipore purification system (Milford, MA, USA) and analytical grade chemicals purchased from commercial sources. Tris (2-Amino-2-(hydroxymethyl)propane-1,3-diol) and sodium bicarbonate were obtained from Calbiochem (Germany), potassium phosphate was from Chemapol (Sweden) and EDTA from Serva Feinbiochimica (Germany). L-DOPA (2-Amino-3-(3,4-dihydroxyphenyl)propanoic acid) was purchased from Aldrich (Germany) and ammonium sulfate from Roth (Germany). L-tyrosine (Y) was obtained from Fluka (Buchs, Switzerland). For chromatography two resins, namely Sephadex gel G-100 and Source 30Q (GE Healthcare, Little Chalfont, UK), were used. Fresh mushrooms were procured from a local market.

2.2. Tyrosinase activity assay

The tyrosinase activity was measured using L-DOPA as a substrate [29]. The enzymatic assay was relied on L-DOPA conversion to dopaquinone by mushroom tyrosinase. For this purpose, the enzyme



activity assay was performed in a 96-well plate. A volume of 200 μL L-DOPA (20 mM) was pipetted to each well over which 20 μL of each eluted fraction was added. The absorbance was recorded every 5 min at 450 nm and 37°C under static conditions. All spectrophotometric measurements were performed using a ModulusTM 96-wells microplate reader (Turner Biosystems, Sunnyvale, USA). The amount of released dopachrome was estimated by using a molar extinction coefficient of 3600 $\text{M}^{-1} \text{cm}^{-1}$ [30].

2.3. TYR extraction protocol

The first step was performed in order to extract the tyrosinase from commercial mushrooms (*Agaricus bisporus*). The enzyme distinguishes by a specificity for tyrosine and therefore could be a potential candidate for quantification of this amino acid in blood samples. A total mass of 150 g of mushrooms was placed in the blender. After grinding, the mushrooms were homogenized using a volume of 300 mL of 50 mM Tris-HCl $p\text{H} = 5.8$ cold buffer on an ice bath. The resulting cooled mixture (maintained on the ice bath) was sonicated using a CPX 130 device (Cole-Parmer Instruments, Illinois, USA). The process parameters (sonication time and temperature) are displayed in Table 1.

Table 1. Parameters of the sonication process

Sonicator parameters			Temperature of the mixture (°C)	
Time (min)	Pulse (s)	Amplitude (%)	Initial	Final
2	1:1	50	5.3	5.8
Pause 5 min				
3	1:1	50	5.3	5.3
Pause 15 min				
3	1:1	50	4.2	4.4
Pause 15 min				
3	1:1	50	4.8	4.9

2.4. Ammonium sulphate precipitation and dialysis

The TYR precipitation by ammonium sulfate has followed using a modified Haghbeen protocol [31]. An amount of 50 g ammonium sulphate was slowly added to 256 mL cooled enzyme-containing supernatant in approximately 90 min. The purpose of this step was to obtain a final concentration of 35% ammonium sulphate, sufficient to precipitate different compounds which increases the effect of matrix. Subsequently, the resulting suspension was centrifuged for 10 min at 5000 rpm and 7°C. The resulted supernatant was used in the next step.

Analogously, an amount of 92 g of ammonium sulfate was added in order to obtain a final concentration of 85%. The obtained slurry was subjected to a new centrifugation step for 30 min at 5000 rpm and 6°C. The supernatant was discarded and precipitate containing TYR was collected.

The resulted precipitate was resuspended with a small amount of 100 mM potassium phosphate buffer ($p\text{H} = 7.00$). The suspension was transferred to a dialysis bag (regenerated cellulose tubular membrane with a 6.0 kDa cut-off, Carl-Roth, Karlsruhe, Germany) which was placed in the same buffer. The entire process was performed at 4°C for 80 h and the solution has been replaced five times. After dialysis, the concentrated mushroom extract was placed in a freezer (-40°C) in order to maintain the tyrosinase integrity.

2.4. Tyrosinase separation by chromatography

2.4.1. Separation by size exclusion chromatography

The dialyzed fraction was applied to the Sephadex gel G-100 column (2.5 x 30 cm, $V_{\text{column}} = 147 \text{ cm}^3$) [32, 33]. The column void volume (70 mL) was determinate using Blue Dextran (2 MDa, Pharmacia, Sweden) as a molecular weight control. The column was pre-equilibrated with the 100 mM potassium phosphate buffer ($p\text{H} = 7.00$). The elution progress was followed (Figure 1). A number of 36 fractions (noted Fr1 to Fr36) were collected in 2 mL eppendorf tubes with a flow rate of 0.05 mL/min. Nine fractions were distinguished by a superior enzymatic activity (Fr11 to Fr19, Figure 2). These fractions were pooled down and used for further purification.

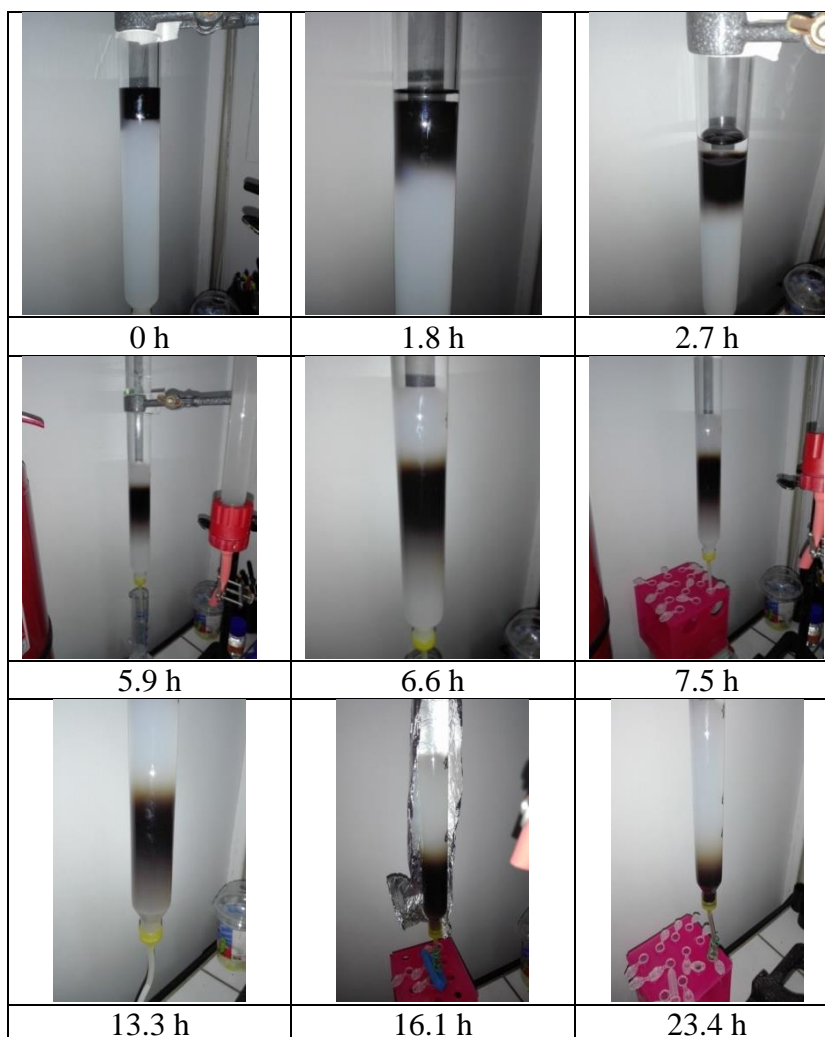


Figure 1. Protein separation by size exclusion chromatography on G-100 Sephadex matrix.

The tyrosinase containing fractions were eluted after almost 24 h

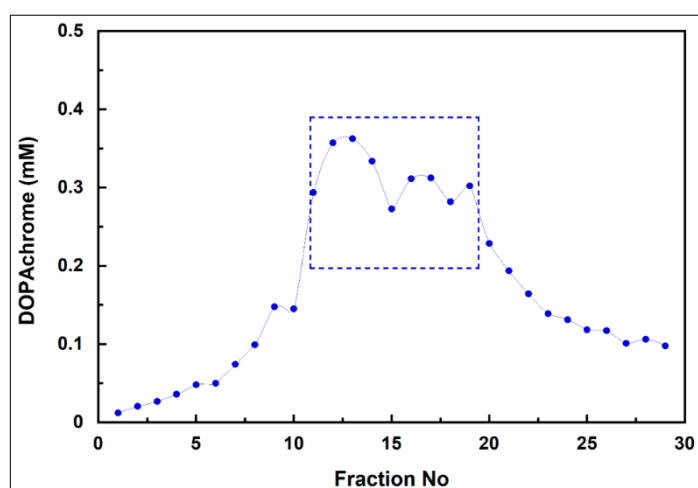


Figure 2. The profile of enzymatic activity of the various fractions eluted from Sephadex G-100 column

2.4.2. Separation by ion exchange chromatography

Anion and cation exchange chromatography is a common separation method used for tyrosinase purification [34-36]. A similar strategy was also used in our study. The protein mixture obtained after separation by gel filtration chromatography was centrifuged. The resulted supernatant was loaded with a rate of 0.7 mL/min on a Source 30Q column (1.0 x 9.0 cm; $V_{\text{column}} = 7.0 \text{ cm}^3$). This column was pre-equilibrated with 50 mL buffer (50 mM potassium phosphate solution, $pH = 7.00$). After extract application, the column was washed with 40 mL of buffer in order to remove non-specifically attached proteins to the strong anion exchanger packed column. A step elution (from 0.1 to 1 M NaCl) gradient was used to purify the enzyme. The eluted samples (5 mL/fraction, Figure 3, inset) were collected in ten 15 mL plastic tubes. The fraction eluted from the second chromatographic step were tested for enzymatic activity (Figure 3).

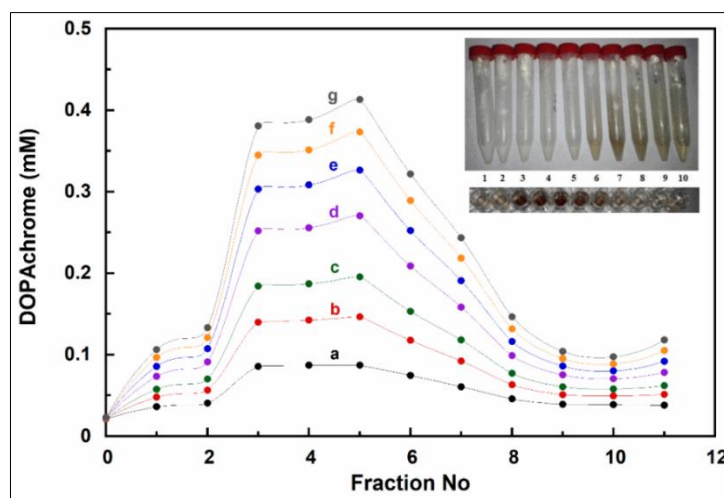


Figure 3. The enzymatic activity profile, expressed as resulted DOPAchrome, for ion exchange chromatography eluted fractions using a salt gradient. The activity was recorded at 0, 5, 10, 20, 30, 40 and 50 min at 450 nm. The fractions 3-7 are distinguished by a higher enzymatic activity

For clarity, the enzymatic profile of tyrosinase as function of NaCl concentration, recorded after 50 min, is illustrated in Figure 4. The fraction with highest activity was eluted at 500 mM NaCl. The fractions with an enhanced enzymatic activity (Fr3 to Fr7) were pooled down and concentrated using a Microcon YM-30 device (Millipore, Bedford, MA) at 6°C and 3000 rpm for 40 min.

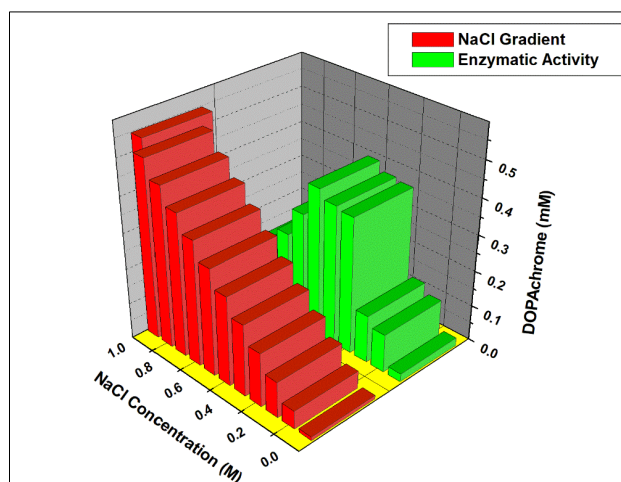


Figure 4. 3D representation of the enzymatic activity elution profile when a step way salt gradient was applied during

2.5 Standard enzymatic activity assay

The enzymatic activity of the purified TYR was tested using different standard solutions of tyrosine (Y), with a concentration varying in the range of 0.01 to 1 mM. The activity assay was performed at 37°C and the absorbance changes were monitored at 450 nm for 90 min. The activity profiles are as function of Y are displayed in Figure 5A. The sensitivity was 250 pMol Y/ μ L and LOD lower than 10 pMol Y/ μ L. The reaction rate was determined for first 30 min using a linear regression profile. The resulted Michaelis Menten parameters are in concordance with previous reported data for L-DOPA and L-dopamine as a substrate [37]. In this assay a Michaelis constant of $265 \pm 32 \mu\text{M}$ was obtained when tyrosine (Figure 5B) was chosen as an enzyme substrate.

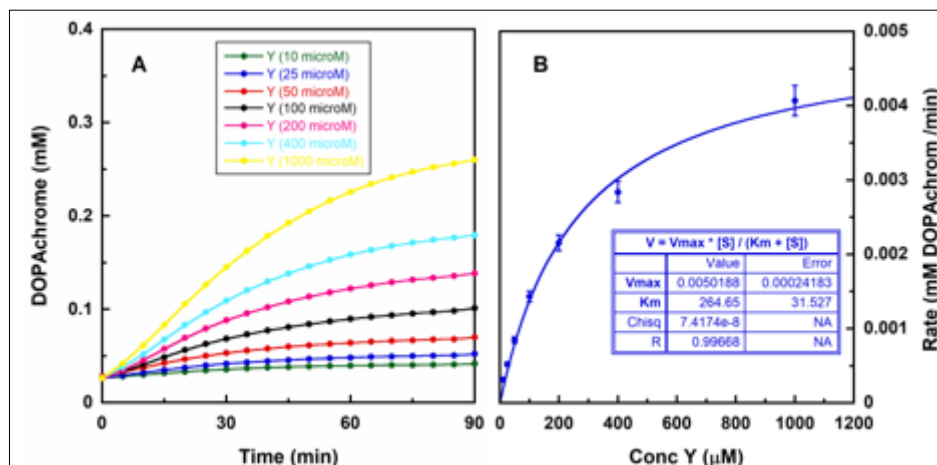


Figure 5. The tyrosinase activity assays profile at various tyrosine concentrations and 37°C (Panel A) and calculated kinetic parameters (Panel B)

2.6. Blood samples

The blood samples were collected in red vacutainers containing a coagulation accelerator additive by venous puncture from hospitalized patients. This study was approved by the Ethics Committee of St. Maria Children Emergency Hospital Iasi, Romania. All relatives of patients signed a written informed consent form in order to donate a single blood sample vial.

The vials were stored immediately in refrigerator and then transported to the lab using an ice box within 1-2 h. The serum was collected after centrifugation for 10 min at 2000 rpm and 6°C. Serum deproteinization was performed using 0.9 M perchloric acid in a volume ratio of 1:1 [38]. The heterogeneous mixture was centrifuged at 10000 rpm for 10 min at 6°C to remove serum precipitated proteins.

In blood serum the Y concentration ranges between 40-90 μM in normal subjects [39], but in the case of phenylketonuria Y concentration decreases dramatically due to the metabolic deficiency. According to Figure 5, a significant aspect of this enzymatic approach is substrate concentration. The data presented here support the assumption that even for normal patients the activity is difficult to assess. Therefore, this drawback could be remediated using the standard addition method (with Y, Table 2).

Table 2. Sample preparation before tyrosinase activity assay

Sample	Control solution (C)	Blank solution (R)
200 μL S	100 μL H ₂ O	100 μL H ₂ O
-	100 μL A	100 μL A
400 μL T ₁	400 μL T ₁	400 μL T ₁
100 μL Y	100 μL Y	100 μL T ₂
40 μL E	40 μL E	40 μL E

where: S-supernatant, T₁-Tris 0.76 M (pH = 8.0), Y-Tyr 1.46 mM (in Tris 0.2 M, pH=5.8), E- purified TYR A- perchloric acid 0.9 M, T₂-Tris 0.2 M (pH=5.8).

Blood samples were collected from the following seven patients (Table 3).

Table 3. Characteristics of the patients from Whom the samples were collected

Sample Code	Age
1	3 years 8 months
2	2 years 4 months
3	4 years 1 month
4	2 years 3 months
5	3 years 6 months
6	5 years 10 months
7	4 years 4 months

2.7. Data analysis

All experimental data were processed using a KaleidaGraph 4.03 software (Synergy Software, Reading, PA, USA) and Origin 6.0 software (OriginLab Corporation, MA, USA)

3. Results and discussions

The tyrosinase from commercial mushrooms (*Agaricus bisporus*) was separated by two chromatographic steps. After second separation (ionic exchange chromatography) the enzyme was concentrated and used for activity assay.

The deproteinized serum samples (Table 3) were mixed with a tyrosine solution according to Table 2. Then, a small aliquot of enzyme was added and the mixture incubated for 14 hours (at 37°C and 300 rpm). After incubation, the absorbance of each sample was recorded at 450 nm. The measurements were performed in triplicate. The absorbances mean values of investigated sample were adjusted according to the mean values of reference and control mixture. The subtracted values were in the range 0.056-0.083. However, in this experiment the initial 770 μM substrate (Y) concentration was taken and an 18.5 folds dilution for deproteinized serum was performed. Thus, according with Figure 5B and smaller added amount of serum derived Y this slightly increase is reasonable. According with these results we suggest further experiments by keeping the initial Y (substrate) concentration in the range of 100-150 μM and concentrate the Tyrosine from serum using an organic solvent (as acetone) in order to concentrate the sample by evaporation after protein precipitation.

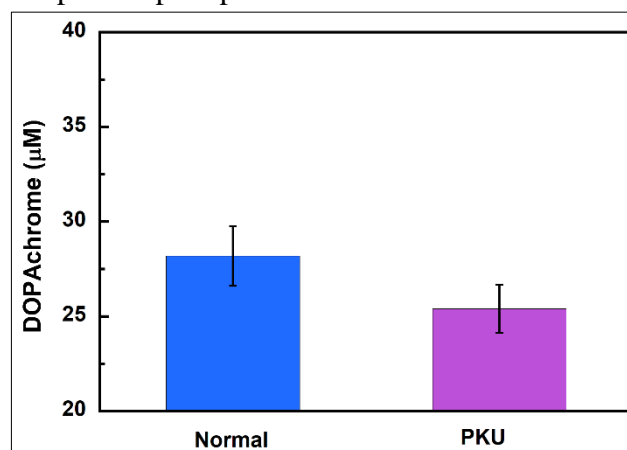


Figure 6. The estimated DOPachrome concentration for both normal and PKU patients

The histogram displayed in Figure 6 indicate the amount of DOPachrome obtained for investigated samples in the presence of purified tyrosinase. The concentration of resulted DOPachrome was slightly higher for normal patients when compared with PKU subjects. This is intriguing since we expected a F:Y ratio higher than three in affected patients. For this reason, this assay is not suitable for quantifying

the level of Y blood level having only a qualitative character. The slightly difference could be attributed to 4-aminobenzoate hydroxylase, an enzyme from *Agaricus bisporus* that convert phenylalanine to tyrosine [38]. To confirm this behaviour a trial experiment was performed. Thus, phenylalanine is not acting as a substrate for tyrosinase alone. However, in the presence of Y (77 μM , at a Y:F 1:2 ratio), the enzyme activity was 10% higher. Interestingly, a considerable activity increase was noticed at a Y:F 1:8 ratio after 23 h of incubation at 22°C. Our data support the idea that F \rightarrow Y conversion is a slowly process mediated by 4-aminobenzoate hydroxylase followed by Y switch in DOPAchrome by mushrooms tyrosinase.

A proposed mechanism of this intriguing process is represented in Figure 7.

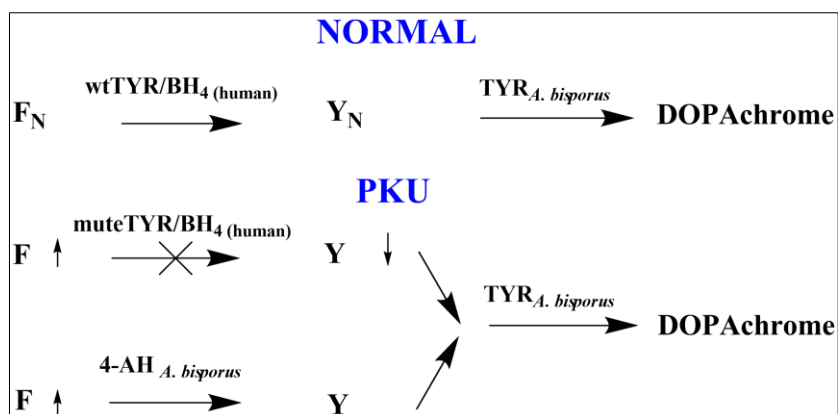


Figure 7. The mechanism of phenylalanine conversion to tyrosine and dopachrome assisted by various enzymes,

where: F – Phenyl alanine; Y - tyrosine; N – normal and PKU affected patients, 4-AH - 4-aminobenzoate hydroxylase, TYR – tyrosinase and BH₄ – tetrahydrobiopterin

The kinetic of 2-aminophenol oxidation in the presence of mushroom tyrosinase was also earlier investigated [40]. Therefore, we suggest a screening of both tyrosinase and 4-aminobenzoate hydroxylase during all purification steps. According with results presented in Figure 5 the method can be safely used for Y concentration higher than 100 μM . Small amounts of 4-aminobenzoate hydroxylase might impair Phe/Y balance and have an undesirable effect on Y detection using the tyrosinase isolated from fungi.

4. Conclusions

The aim of this study was to quantify the concentration of tyrosine in the blood of patients with PKU by a colorimetric enzymatic method. The enzyme used in this determination was TYR, an enzyme extracted from commercially mushrooms (*Agaricus bisporus*). Two chromatographic steps (molecular exclusion and ionic exchange chromatography) were used during the enzyme purification process. The enzyme was active and was successfully used for reasonable concentrations of tyrosine (above 100 μM).

Quantification of tyrosine concentration in serum from patients (PKU, M) by this enzymatic method was extremely difficult. Subsequent studies will use a different protein precipitation agent in the sample processing step and envisage enzyme's contaminants removal. Moreover, future efforts should be undertaken in order to discover a new cheaper enzymatic system or to develop a more sensitive method for tyrosine quantification. Alternative methods are mandatory for an earlier and faster screening of PKU patients.

References

1. WILLIAMS, R.A., MAMOTTE, C.D.S., BURNETT, J.R., Phenylketonuria: an inborn error of phenylalanine metabolism, *Clin. Biochem. Rev.*, **29**(1), 2008, 31-41.

2. FERNSTROM, J.D., FERNSTROM, M.H., Tyrosine, Phenylalanine, and Catecholamine Synthesis and Function in the Brain, *J. Nutr.*, **137**(6), 2006, 1539-1547.
3. SCRIVER, C., KAUFMAN, S., *The Metabolic and Molecular Bases of Inherited Disease* (Eighth Edition), McGraw-Hill, New-York, 2001, 1667-1724.
4. JANSSEN, P.A.J., LEYSEN, J.E., MEGENS, A.A.H. P., AWOUTERS, F.H.L., Does phenylethylamine act as an endogenous amphetamine in some patients?, *Int. J. Neuropsychopharmacol.*, **2**(3), 1999, 229–240.
5. GHOZLAN, A., VAROQUAUX, O., ABADIE, V., Is monoamine oxydase-B a modifying gene and phenylethylamine a harmful compound in phenylketonuria?, *Mol. Genet. Metab.*, **83**(4), 2004, 337–340.
6. CLEMENS, P.C., SCHUNEMANN, M.H., HOFFMANN, G.F., KOHLSCHUTTER, A., Plasma concentrations of phenyllactic acid in phenylketonuria, *J. Inherit. Metab. Dis.*, **13**(2), 1990, 227–228.
7. SARKISSIAN, C.N., SCRIVER, C.R., MAMER, O.A., Measurement of phenyllactate, phenylacetate, and phenylpyruvate by negative ion chemical ionization-gas chromatography/mass spectrometry in brain of mouse genetic models of phenylketonuria and non-phenylketonuria hyperphenylalaninemia, *Anal. Biochem.*, **280**(2), 2000, 242–249.
8. HARDING, C.O., GILLINGHAM, M.B., HAMMAN, K., CLARK, H., GOEBEL-DAGHIGHI, E., BIRD, A., KOEBERL, D.D., Complete correction of hyperphenylalaninemia following liver-directed, recombinant AAV2/8 vector-mediated gene therapy in murine phenylketonuria, *Gene Ther.*, **13**, 2006, 457–462.
9. KAUFMAN, S., A model of human phenylalanine metabolism in normal subjects and in phenylketonuric patients, *Proc. Natl. Acad. Sci. USA*, **96**(6), 1999, 3160-3164.
10. CRICIOTOIU, O., STANCA, D.I., GLAVAN, D.G., MIREA, C.S., MITA, A., CALBOREAN, V., GHEORMAN, V. UDRISTOIU, I., DIJMARESCU, A.L., DAVITOIU, D.V., GHEONEA, D.I., Statistical Analysis Between the Routes of Administration of L-Dopa and Digestive Dysfunctions in Parkinson Disease, *Rev. Chim.*, **70**(4), 2019, 1403-1405.
11. SLOMINSKI, R. M., ZMIJEWSKI, M. A., SLOMINSKI, A. T., The role of melanin pigment in melanoma, *Exp. Dermatol.*, **24**, 2015, 258–259.
12. HADDAD, F., SAWALHA, M., KHAWAJA, Y., NAJAR, A., KARAMAN, R., Dopamine and Levodopa Prodrugs for the Treatment of Parkinson's Disease, *Molecules*, **23**(1), 2018, E40.
13. NICOLAE, I., ENE, C.D., SCHIPOR, S., TAMPA, M., MATEI, C., GEORGESCU, S.R., Dopamine-chemical Mediator in Atopic Dermatitis, *Rev. Chim.*, **64**(10), 2013, 1201-1206.
14. NORRIS, D.O., CARR, J.A., *Vertebrate Endocrinology*, Academic Press, 2013, 41–91.
15. YAMAUCHI, K., *Handbook of Hormones. Comparative Endocrinology for Basic and Clinical Research*, Academic Press, 2016, 498–500.
16. CHRISTENSEN, R., KOLVRAA, S., JENSEN, T.G., Manipulation of the Phenylalanine Metabolism in Human Keratinocytes by Retroviral Mediated Gene Transfer, *Cells Tissues Organs*, **179**(4), 2005, 170-178.
17. MIHALI, C.V., LADASIU CIOLACU, C.F., FRENTESCU, L., PETRESCU, C.M., MANDRUTIU, I., BECHET, D., NISTOR, T., ARDELEAN, A., BENGA, G., Determination of plasma phenylalanine concentration by two dimensional thin layer chromatography and high performance liquid chromatography in relation with diagnosis of phenylketonuria, *Studia UBB Chemia*, **58**(4), 2018, 79-84.
18. ASHE, K., KELSO, W., FARRAND, S., PANETTA, J., FAZIO, T., DE JONG, G., WALTERFANG, M., Psychiatric and Cognitive Aspects of Phenylketonuria: The Limitations of Diet and Promise of New Treatments, **10**, Art. No.561.
19. ROCHA, J.C., MARTEL, F., Large neutral amino acids supplementation in phenylketonuric patients, *J. Inherit. Metab. Dis.*, **32**(4), 2009, 472-480.
20. PIETZ, J., KREIS, R., RUPP, A., MAYATEPEK, E., RATING, D., BOESCH, C., BREMER H.J., Large neutral amino acids block phenylalanine transport into brain tissue in patients with phenylketonuria, *J. Clin. Invest.*, **103** (8), 1999, 1169-1178.



21. ADLER-ABRAMOVICH, L., VAKS, L., CARNY, O., TRUDLER, D., MAGNO, A., CAFLISCH, A., FRENKEL, D., GAZIT, E., Phenylalanine assembly into toxic fibrils suggests amyloid etiology in phenylketonuria, *Nat. Chem. Biol.*, **8**, 2002, 701-706.
22. HAGEN, M.E.K., PEDERZOLLI, C.D., SGARAVATTI, A.M., BRIDI, R., WAJNER, M., WANNMACHER, M.D., WYSE, A.T.S., DUTRA-FILHO, C.S., Experimental hyperphenylalaninemia provokes oxidative stress in rat brain, *Biochim. Biophys. Acta*, **1586**(3), 2002, 344-352.
23. DUARTE, J.M., SCHUCK, P.F., WENK, G.L., FERREIRA, G.C., Metabolic Disturbances in Diseases with Neurological Involvement, *Aging Dis.*, **5**(4), 2013, 238-255.
24. DYER, C.A., Comments on the neuropathology of phenylketonuria, *Eur. J. Pediatr.*, **159**(S2), 2000, 107-108.
25. HUTTENLOCHER, P.R., The neuropathology of phenylketonuria: human and animal studies, *Eur. J. Pediatr.*, **159**(2), 2000, 102-106.
26. ATES, S., DOGAN, N.S., *Turk. J. Biochem.*, Properties of Immobilized Phenylalanine Ammonia Lyase and Investigation of its Use for the Prediagnosis of Phenylketonuria, **35**(1), 2010, 58-62.
27. HAGHBEEN, K., RASTGAR JAZII, F., KARKHANE, A.A., SHAREEFI BOROJERDI, I., Purification of tyrosinase from edible mushroom, *Iran. J. Biotechnol.*, **2**(3), 2004, 189-194.
28. VELICHKOVA, P., MARINKOVA, D., YANEVA, S., YOTOVA, L., Isolation and purification of tyrosinase from different plant sources, *Annual of Sofia University*, **100**(4), 2015, 70-75.
29. LAI, X., SOLER-LOPEZ, M., WICHERS, H.J., DIJKSTRA, B.W., Large-scale recombinant expression and purification of human tyrosinase suitable for structural studies, *PLoS One*, **11**(8), 2016, 1-16.
30. NEEDLY, E., FRITCH, G., FULLER, A., WOLFE, J., WHIGHT, J., Various in IC₅₀ values with purity of mushroom tyrosinase, *Int. J. Mol. Sci.*, **10**, 2009, 3811-3823.
31. WINGFIELD, P. T., Protein precipitation using ammonium sulfate, *Curr. Protoc. Protein. Sci.*, 2016, Supplement 84, A.3F.1-A.3F.9.
32. REILAND, J., Gel Filtration, *Meth. Enzymol.*, **22**(C), 1971, 287-321.
33. TSUJI, H., OGAWA, T., BANDO, N., SASAOKA, K., Purification and properties of 4-aminobenzoate hydroxylase, a new monooxygenase from *Agaricus bisporus*, *J. Biol. Chem.*, **261**(28), 1986, 13203-13209.
34. MACHOLD, C., SCHLEGL, R., BUCHINGER, W., JUNGBAUER, A., Matrix assisted refolding of proteins by ion exchange chromatography, *J. Biotechnol.*, **117**(1), 2005, 83-97.
35. ZHANG, X., FLURKEY, W. H., Purification and partial characterization of tyrosinase isoforms from cap flesh of *Portabella* mushrooms, *J. Food Biochem.*, **23**(1), 1999, 95-108.
36. PANAINTE, A. D., MORARIU, I. D., BIBIRE, N., VIERIU, M., TANTARU, G., LUCA, A. C., APOSTU, M., Studies on chromatographic fractioning by cations exchangers of a bovine hemoglobin hydrolysate, *Rev. Chim.*, **69**(10), 2018, 2794-2798.
37. NAIRN, R., CRESSWELL, W., NAIRN, J., Mushroom Tyrosinase: A Model System to Combine Experimental Investigation of Enzyme-Catalyzed Reactions, Data Handling Using R, and Enzyme-Inhibitor Structural Studies, *Biochem. Mol. Biol. Edu.*, **43**(5), 2015, 370-376.
38. CHUANG, C. K., WANG, T. J., YEUNG, C. Y., HSIEH, W. S., LIN, D. S., HO, S. C., LIN, S. P., Interference and blood sample preparation for a pyruvate enzymatic assay, *Clin. Biochem.*, **39**(1), 2006, 74-77.
39. GRUNERT, S.C., BRICHTA, C.M., KREBS, A. CLEMENT, H.W., RAUH, R. FLEISCHHAKER, C., HENNIGHAUSEN, K., SASS, J.O., SCHWAB, K.O., Diurnal variation of phenylalanine and tyrosine concentrations in adult patients with phenylketonuria: Subcutaneous microdialysis is no adequate tool for the determination of amino acid concentrations, *Nutr. J.*, **12**(1), 2013, 1-12.
40. PUIU, M., RADUCAN, A., OANCEA, D., The inhibition of 2-aminophenol oxidation in the presence of mushroom tyrosinase, *Rev. Chim.*, **55**(6), 2004, 426-429.

Manuscript received: 11.12.2019