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Kinetic investigation and tyrosinase inhibition activity of peptide analogues of galanthamine

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The hyper production of melanin is a reason for malignant melanoma, the most life-threatening skin cancer. Recently, tyrosinase inhibitors attract a lot of attention because of their ability to influence the activity if this key enzyme. Such kind of compounds is increasingly used as ingredients in cosmetic creams and products. Peptides containing aspartic or glutamic acid residues usually do not bind very well to tyrosinase. Strong tyrosinase-binding peptides always contain one or more arginine residues, often in combination with phenylalanine, while lysine residues can be found equally among nonbinding peptides as well as moderate tyrosinase-binding peptides. The presence of the hydrophobic, aliphatic residues valine, alanine or leucine appears to be important for tyrosinase inhibition. Therefore, good tyrosinase inhibitory peptides preferably contain arginine and/or phenylalanine in combination with valine, alanine and/or leucine. A special place is given to peptides, because of their good bioavailability and low or lack of toxicity.

Herein, we report the kinetic investigation, inhibitory activity and IC_{50} of two peptide amide of galanthamine Boc-Asp(norGal)-Asp-Leu-Ala-Val-NH-Bzl and Boc-Asp(norGal)-Asp-Leu-β-Ala-Val-NH-Bzl.

Key words: peptides, enzymes, inhibitors; pharmaceutical application

INTRODUCTION

Tyrosinase (EC 1.14.18.1.) is a metaloenzyme containing Cu^{+2} as a cofactor in the active side that catalysis two districts reaction of melanin synthesis, the hydroxylation of a monophenol and the bioconversion of o-diphenol to the corresponding o-quinone [1]. The latter undergoes several reactions to form melanin (figure 1).

Currently a great interest is the involvement of melanin (one of the most common pigments including in human skin) in several dermatological disorders. The hyper production of melanin is a reason for malignant melanoma, the most life-threatening skin cancer. Recently, tyrosinase inhibitors attract a lot of attention because of their ability to influence the activity if this key enzyme. Such kind of compounds is increasingly used as ingredients in cosmetic creams and products [2]. A special place is given to peptides, because of their good bioavailability and low or lack of toxicity [3]. Herein, we report the kinetic investigation and inhibitory activity of two peptide amide of galanthamine Boc-Asp(norGal)-Asp-Leu-Ala-Val-NH-Bzl and Boc-Asp(norGal)-Asp-Leu-β-Ala-Val-NH-Bzl.

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MATERIALS AND METHODS

Tyrosinase inhibition activity

All kinetic investigations and IC_{50} determinations were done using an **optical biosensor** with tyrosinase from mushrooms (EC 1.14.18.1), immobilized **onto** hybrid membranes synthesized by sol-gel technology. Synthesis of used membranes containing cellulose acetate propionate with high molecule weight (~25 000) (CAP), methyl triethoxysilane (MTES) and co-polymer of acrylamide/acrylonitrile is described in [4]. The quantity of protein immobilized onto the membranes, was determine using Lowry's methodology [5]. Initially, the activity of the immobilized tyrosinase was measured without presence of inhibitor. Diphenolase activity was determined spectrophotometrically with 10 mM substrate L-DOPA (L-3,4-dihydroxyphenylalanine) as a substrate, at 25 °C, using spectrophotometer with optical fibers (AvaSpec, Avantes, USA).

The diphenolase activity does not show any lag period. The dopachrome assay was performed. The increase in absorption at 475 nm, due to the formation of dopachrome (ε 475 = 3 600 $M^{-1}cm^{-1}$), was monitored as a function of time. The activity is expressed as mole of L-DOPA oxidized per minute.

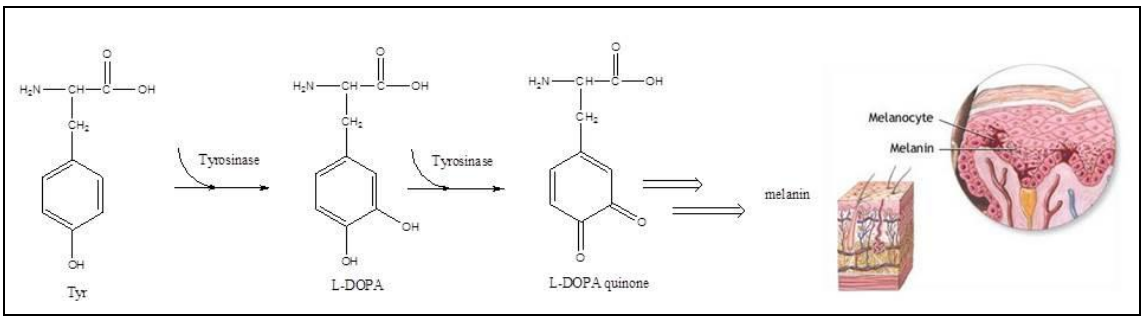

Fig. 1. Two stages of tyrosinase participation in melanin synthesis

1 ml of 0.001M potassium phosphate buffer (pH=7) and 1 ml $13.3x10^{-5}$ M L-DOPA were stirring. Further, synthesized peptides at different concentrations from 5 μM to 100μM were diluted in 1.0 ml of 0.001M potassium phosphate buffer (pH=7). 50mg of membranes with the immobilized enzyme were added directly to the solution and were incubated together for 30 min at 25° C. The membranes with immobilized tyrosinase, were moved out from the solution and the residual activity was measured following the procedure according to Worthington [6].

Peptide inhibitors were synthesized according to methodology described in [7]. They all are amides or esters of natural galanthamine with following structures:

Boc-Asp-(norGal)-Asp-Leu-Ala-Val-NH-Bzl – inhibitor 1- (**I_1**);

Boc-Asp-(norGal)-Asp-Leu-β-Ala-Val-NH-Bzl – inhibitor 2- (**I_2**).

The inhibitory effects of all the analyzed Tyrosinase inhibitors was calculated by measuring the difference in the enzyme activity before and after incubation with inhibitor. The measurement was done at 460 nm for 5 min.

The inhibition percentage was calculated according to equation.

Inhibition (%) = $[(E_0 - Ei)/E_0]*100$,

Where E_0 is the initial inhibited sensor activity and E is the inhibited sensor activity. The sensitivity of the biosensor toward tyrosinase was measured.

RESULTS AND DISCUSSION

A lot of scientific groups are investigated peptides with different structures as potential inhibitors of tyrosinase in order to estimate role of different amino acids for inhibitory activity. Schurink et al. reveal that strong peptide based tyrosinase inhibitors always contain one or more arginine residues, often in combination with phenylalanine. In addition they prove that the presence of hydrophobic, aliphatic residue like valine, alanine or leucine is key factor for tyrosinase inhibitory potential [3]. In contrary, Noh et al. describe series of 22 tripeptides combined with kojic acid where all compounds with strong inhibitory activity against tyrosinase contain minimum one hydrophilic amino acid [8]. Many researchers studied inhibitors from natural sources such as silk, yogurt and more [9-12]. A lot of other examples appear in the scientific literature but finally one is clear that still there is no exactly defined structure-activity relationship for peptide containing molecules and their anti-tyrosinase activity. That's why we studied inhibition activity of two peptide containing analogues of norgalanthamine including combination of hydrophobic (Leu, Ala/β-Ala, Val) and one hydrophilic (Asp) residue. C-terminus of aim peptides is modified as benzylamide in order to have hydrophobic properties. In addition, both aim peptide analogues differ by the presence of Ala or β-Ala in their structure in order to evaluate their influence on inhibitory activity.

All investigations for inhibitory activity of both compounds are made using optical biosensor containing tyrosinase immobilized on hybrid matrix. Initially, we made the calibration curves with inhibitors.

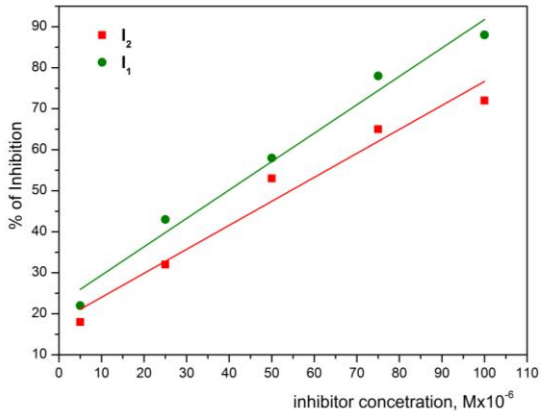

Fig. 2. Calibration curves for free and immobilized tyrosinase with the peptide inhibitors I_1, I_2

Table 1. Ki value for I_1 and I_2 for free and immobilized tyrosinase

Inhibitor concentration, $Mx10^{-6}$	Ki free tyrosinase, $Mx10^{-6}$		Ki immobilized tyrosinase, $Mx10^{-6}$	
	I_1	I_2	I_1	I_2
5	0.65	0.29	1.84	1.25
25	2.75	1.61	3.11	3.47
50	4.82	3.99	5.68	6.36
75	5.92	5.95	7.56	7.09

They are illustrated on Figure 2 in the presence of tyrosinase at $13.3x10^{-5}$ M concentration. The calibration curves exhibited linear response in the concentration range $5.10^{-6} \div 100.10^{-6}$M.

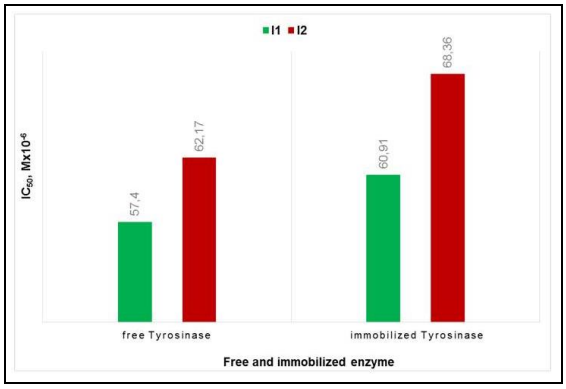

Fig. 3. IC_{50} determined for I_1 and I_2 for free and immobilized tyrosinase

After drawing the calibration curves, the IC_{50} values for the used inhibitors are determined and they are shown in figure 3.

The results shown IC_{50} for free enzyme is $57.4.10^{-6}$ and $62.17.10^{-6}$M for I_1 and I_2, respectively. IC for immobilized tyrosinase was calculated $60.91.10^{-6}$ and $68.36.10^{-6}$ M for I_1 and I_2.

The values of Ki for both inhibitors concentrations for free and immobilized tyrosinase are presented at Table 1. The results showed that inhibitors act as uncompetitive for tyrosinase.

After the spectrophotometric determination of inhibitory activity of peptide derivatives of galanthamine, samples containing substrate L-DOPA, enzyme and inhibitor were stored in the dark at t = 4 °C for 30 days. Periodically on the first, 15th and 30th day the samples are measured to determine the effect of the inhibitors in time and final product formation - melanin. The results of the tests show that both inhibitors have excellent inhibitory properties against tyrosinase, by suppressing formation of melanin for a period of - longer than 15 days.

CONCLUSIONS

The results from our study shows that inhibitors of two peptide amide of galanthamine Boc-Asp(norGal)-Asp-Leu-Ala-Val-NH-Bzl and Boc-Asp(norGal)-Asp-Leu-β-Ala-Val-NH-Bzl acting uncompetitive against tyrosinase. The obtained peptides could find potential applications into the medical cosmetology and prevention of diseases related to pigmentation disorders.

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ИЗСЛЕДВАНЕ ИНХИБИТОРНАТА АКТИВНОСТ ВЪРХУ ТИРОЗИНАЗА НА ПЕПТИДНИ АНАЛОЗИ НА ГАЛАНТАМИН

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(Резюме)

Хипер производството на меланин е причина за възникването на малигнен меланом – вид агресивен рак на кожата. Хипофункцията на меланин в организма се отключва все по-често срещаното заболяване, свързано със загуба на пигментация – витилиго. В последните години инхибиторите на тирозиназата както и нейните активатори привличат вниманието на изследователите, заради способността да се повлиява действието на ензима. Този вид съединения все по-често се включват като съставка в козметични кремове и продукти

Пептиди, съдържащи остатъци на аспаргинова и глутаминова киселини, обикновено не се свързват добре с тирозиназата. Ефективно свързване се постига с пептиди, съдържащи един или повече аргининови остатъци, често заедно с фенилаланин. От друга страна лизиновите остатъци имат двойствен характер и се срещат както при несвързващите, така и при умерено свързващите тирозиназата пептиди. Присъствието на хидрофобните алифатни остатъци валин, аланин или левцин са важни за инхибирането на тирозиназата. Следователно, добри тирозиназни инхибитори са пептидите съдържащи в състава си аргинин и/или фенилаланин заедно с валин, аланин и/или левцин. Инхибиторите с пептидна структура са особено подходящи за приложение поради тяхната натурална природа и ниска или отсъстваща токсичност.

В настоящата работа докладваме изследването на инхибиторната активност и IC_{50} на два пептида амидни аналози на галантамин Boc-Asp (norGal) -Asp-Leu-Ala-Val-NH-Bzl и Boc-Asp (norGal) -Asp-Leu-β- Ala-Val-NH-Bzl.