Ph.D. Thesis

Inhibition of carbonhydrate cleaving enzymes in STZ induced diabetes mellitus model

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2018

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2 Introduction

The treatment of hyperglycaemia is crucial in the management of metabolic syndromes such as type II diabetes. The α-amylase, and α-glucosidase as digestive enzymes have an essential role in glucose release taking part in dietary polysaccharides hydrolysis. These enzymes have important role in diabetes research, because they are potential targets for antidiabetic drug research. It is a commonly accepted strategy to manage hyperglycaemia by the inhibition of α-amylase and α-glucosidase. The inhibition of these enzymes postpones remarkably the adsorption of glucose along with the postprandial hyperglycaemia. The acarbose is widely applied antidiabetic drug that inhibit pancreatic α-amylase and intestinal α-glucosidase. Although it is very effective, but it has several unpleasant gastrointestinal side effects. This is the reason that there is an increased demand for new molecules possessing less side effects. Several clinical studies, dealing with the postprandial glycaemic investigations, have demonstrated the effectiveness of the

food polyphenols in the treatment of high blood glucose levels. The berries are rich in these compounds that wild strawberry, bilberry and blueberry were chosen to study the inhibition assays of glycolytic enzymes and to reduce postprandial hyperglycaemia.

3 Aims

Nowadays, the type II diabetes is the most commonly occurring disorder in civilisation, which could be treat successfully using medicinal herbs. To facilitate the possibility of practical applicability of the medicinal herbs the aims of my PhD thesis were the followings:

1. Preparation of several medicinal herbs extracts containing bioactive components and assay of their *in vitro* anti-diabetic activities.
2. Development of a novel HPLC method for the measurement of *in vitro* α-amylase activities.
3. Determine the effects on cells of the selected medicinal plant extracts using cell lines.

4. Mapping the possible bioactive components with mass spectrometric investigations.
5. Testing of the herb extracts *in vivo* in STZ induced and HFHS diet treated diabetic rats.

4 Materials and methods

1. Sample preparation procedures:
    a. Selection of plants, aquatic extraction of herbs and lyophilization.
    b. Tannin precipitation from the plant extracts.
2. Examination of the effect of plant extracts on the glycoside-hydrolase enzymes.
    a. Development of HPLC method for the measurement of α-amylase inhibition, synthesis and purification of the substrate.
    b. Determination of α-amylase inhibition of the plant extracts using the developed method.

c. Determination of α-glycosidase inhibition of the plant extracts with spectrophotometric method.

3. Measurement of the antioxidant capacity of the plant extract.
4. Investigation of the extracts with mass spectrometric technique (MALDI-TOF).
5. Cell based bioactivity assays:
    a. Cytotoxicity assay on H9c2 cells
    b. Cytoprotecting assay with RTCA-SP system.
6. *In vivo* assays:
    a. Applying of chemically induced diabetes model for the test of extracts.
    b. Applying of HFHS dietic induced model for the test of extracts.

5 Results

5.1 Development of α-amylase inhibition measurement method

During the method development the 2-chloro-4-nitrophenyl-β-D-maltoheptaoside (CNP-G7) substrate was synthetized, which was purified via preparative chromatographic technique. The purity of the final product was checked with liquid chromatography showing the retention time for CNPG-7 6.7 min and for purity 96.9%. For the comprehensive confirmation of our results mass spectrometric examinations were also carried out in positive ionization mode with MALDI-TOF MS technique. The *m/*z values of potassium and sodium adducts characteristics for the CNPG-7 appeared in the purified substrate on the spectra without any interfering components.

5.2 Effects of plant extracts to the glycoside-hydrolase enzymes

During the pretreatment the aquatic extraction of plant material was carried out followed by lyophilization. The dried plant samples were dissolved in water and after the centrifugation their effects on both α-amylase and α-glucosidase were determined. After the measurements three plants were selected with the 8.84±2.8 μg/ml, 25±8 μg/ml and 27.27±9 μg/ml IC_{50} values for wild strawberry, blueberry and blackberry, respectively. The effects of the mixture created from these plants in equal ratio was also tested and the observed IC_{50} values were 11.2 μg/ml and 15.3 μg/ml in the case of α-glucosidase and α-amylase, respectively.

The enzyme inhibition examinations were also carried out after the precipitation of tannins from the mixture causing only 7.2% and 14.9% loss of the original inhibition values for α-amylase and α-glucosidase, respectively. However, the observed inhibition values remained at high level, thus it could be concluded that the inhibition caused not only from the tannin content.

5.3 Antioxidant capacity of the selected plant extracts

The abilities of the plant metabolites to inhibit the releasing of free radicals and the connected harmful side effects termed as antioxidant capacity. The measurement of antioxidant capacity were carried out in each plant extract. The C-vitamin equivalent antioxidant capacities of blueberry, wild strawberry and blackberry were 809.15±68.27 μg/mg, 490.47±56.97 μg/mg and 366.32±42.67 μg/mg, respectively.

5.4 Mass spectrometric analysis of the selected plant extract

During the measurement of the plant extracts, the metabolites were identified in the samples based on their kationized – protonated, potassium and/or sodium adducts – quasi molecular ions. If all of three molecular ions were detected including *m/z* values of $[M+H]^+$, $[M+K]^+$ and

$[M+Na]^+$, the chemical composition s were detected with the Metabolomics Workbench Databank. Remarkable part of the detected metabolites is belonging to the group of flavonoids, whose biological activities are known from more aspects.

5.5 Cytotoxic and citoprotective effects of the selected plant extracts

The possible cytotoxic effects of the plant extracts were tested by MTT-assay, while the cytoprotective effects were measured with real-time cell electronic sensing system (RT-CES) using H9c2 embryonal rat heart cell lines. According to the results of the performed examinations, it could be concluded that the applied plant extracts cannot be considered toxic in the used *in vitro* setup. However, in the cytoprotection experiments the harmful effects of the hydrogen-peroxide as stressor could not be protected via none of the extracts.

5.6 Analysis of the extracts in *in vivo* diabetes mice model

For the first *in vivo* model STZ induced CD1mice were used. Five days after the STZ treatment, the blood glucose level was significantly improved due to the starch administration. The effects of both the acarbose and plant extract treated mice were similar, because in both cases the blood glucose concentrations reduced. For further diabetic mice model C57B16 mice were used. This type of mice susceptible to diet induced obesity. The control group for type II diabetes model had the same age and fed with normal diet. This experimental group showed no difference after administration of starch and plant extracts or acarbose. The starting blood glucose level of this group were notably lower compared to the diabetic mice. After administration of starch followed by acarbose or plant extract the blood glucose level remained stable throughout the experiment in both cases.

6 Conclusions

1. Eight plants were selected based on literature whose glycoside-hydrolase activity were teted *in vitro*. The plant extracts inhibited the α-amylase with dose dependent manner. The related IC_{50} value of each plants were determined. The strawberry extract showed the lowest value while the IC_{50} value of blueberry and raspberry were significantly higher.

2. Novel HPLC method was developed for the sensitive analysis of α-amylase. The application of HPLC makes it possible to separate components and analyse their quantity as well as the reaction rate could be calculated. A new synthetic substrate was applied that forms stronger bonds with the active centrum of amylase, consequently it models well the natural environments. The gained β-configuration within the synthesis defend the substrate against the cleavage of amylase, thus the

chromophore group remains on the substrate causing the specificity of the detection. The purity of the substrate was determined by liquid chromatography and MALDI-TOF MS.

3. Before the animal experiments to check the effects of the plant extracts on cells in vitro cytotoxic and cytoprotecting tests were applied. The results showed that none of the examined plant had any cytotoxic effect in the investigated concentrations range.
4. The components of plants were analysed with mass spectrometric methods. Using the MALDI-TOF MS results, the formula of a lot of compound were determined and their potential names were determined according to the literature. Based on the results the extracts contained high amount of polyphenolic substances, from which the most important could be the tannins, ellagic tannins, flavonoids and its derivatives.
5. During *in vivo* experiments the plant extracts were tested in both diabetic mice models. The chemically induced diabetes model is an easy and

cost-effective method for studying the pathogenesis of type I diabetes, thus this method was applied on CD1 mice with STZ induction. To test the model type II diabetes, HFHS diet induced, pre-diabetic obese model was applied. For these experiments, C57BL6 mice were used that are susceptible to the diet induced diabetic conditions. In both models the blood glucose level was examined by tail blood. The results showed that the applied plant extracts effectively attenuate postprandial hyperglycaemia in normal and pre-diabetic as well as STZ induced mice. The utilization of mixture of the extracts of the three plants confirmed the applicability of these plants in the treatment of the postprandial hyperglycaemia by the inhibition of α-amylase and α-glucosidase. Furthermore, it could be used safely, because none of cytotoxic effects were observed. The results of our study suggests, involving the *in vitro* and *in vivo* examinations, that the anti-hyperglycaemic effect of the plant extracts are in correlation with the inhibition of α-amylase and α-glucosidase. The

extracts are not toxic and there are no obvious side-effects, consequently it can be aimed as complementer treatment of type II diabetes or metabolic syndrome.

7 Results summarized in the Ph. D. thesis were published in the following articles

MTMT identifier: 10057721

Publications in referred journals

HPLC method for measurement of human salivary α-amylase inhibition by aqueous plant extracts.

Takács, I., Takács, Á., Pósa, A., Gyémánt, Gy. Acta Biologica Hungarica, 2017; 68(2):127-136. doi: 10.1556/018.68.2017.2.1; Q3; IF 0.581

Simple ITC method for activity and inhibition studies on human salivary α-amylase

Lehoczki, G., Szabó, K., Takács, I., Kandra, L., Gyémánt, Gy. Journal of Enzyme Inhibition and Medicinal Chemistry, 2016; 31,(6):1648-1653., Q2 *IF*: 4.293

Conference abstracts summarizing the results of the Ph. D. thesis

Investigation of α-amylase inhibitory activities of herbal extracts with a HPLC-based assay

Takács, I., Gyémánt, Gy., Boros, K., Hohmann, J., Csupor, D. Planta Medica, 2015; 81(16) 1520 DOI: 10.1055/s-0035-1565726

Anti-Amylase and antifungal effect of common herbs and spices

Lehoczki, G., Kovács, R., Takács, I., Pető, K., Gyémánt, Gy. 8th Central European Conference "Chemistry towards Biology" 28th August – 1st September 2016 Brno, Czech Republic Published by University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic ISBN 978-80-7305-777-0

Új HPLC eljárás az alfa-amiláz aktivitás és gátlás mérésére

Takács, I., Pósa, A., Szekeres, A., Endre, G., Gyémán, Gy. 23rd International Symposium on Analytical and

Environmental Problems October 9-10, 2017 University of Szeged, Department of Inorganic and Analytical Chemistry Szeged Hungary

Társszerzői nyilatkozat

Jelölt neve: Takács István

Doktori iskola : SZTE TTIK , Biológia Doktori Iskola

Közlemény címe: ***HPLC method for measurement of human salivary α-amylase inhibition by aqueous plant extracts.***

Szerzők:Takács István, Takács Ákos, Pósa Anikó és Gyémánt, Gyöngyi

Megjelenés helye, ideje : Acta Biologica Hungarica, (2017) 68 (2). pp. 127-136. ISSN 0236-5383

Nyilatkozat:

Alulírottak kijelentjük, hogy a fenti közleményben megjelent és a jelölt által a Szegedi Tudományegyetemre benyújtott Ph.D. értekezésben felhasznált tudományos eredmények eddig nem szerepeltek más Ph.D. értekezés tudományos eredményei között. Tudomásul vesszük, hogy a fenti tudományos eredmények nem szerepelhetnek más Ph.D. értekezés eredményei között.

Takács István (jelölt) .....

Takács Ákos (társszerző) .....

Dr.Pósa Anikó (témavezető, társszerző) .....

Szeged, 2017. 10. 03.

Dr. Gyémánt Gyöngyi (társszerző) .....

Debrecen, 2017.10.03.

Társszerzői nyilatkozat

Jelölt neve: Takács István Gábor

Doktori iskola : SZTE TTIK , Biológia Doktori Iskola

Közlemény címe: **Simple ITC method for activity and inhibition studies on human salivary α-amylase**

Szerzők: Gábor Lehoczki, Kármen Szabó, István Takács, Lili Kandra, Gyöngyi Gyémánt
Megjelenés helye, ideje: Journal of Enzyme Inhibition and Medicinal Chemistry, 2016, Volume 31, Issue 6, 1648-1653.

Alulírottak kijelentjük, hogy a fenti közlemény első szerzős közleményként került megadásra Lehoczki Gábor benyújtott PhD dolgozatához. Takács István hozzájárulása meghatározó volt az enzimreakciók követésére szolgáló HPLC módszer kidolgozásában és alkalmazásában, ami nem szerepel új tudományos eredményként Lehoczki Gábor tézisei között.
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Takács István (jelölt) .....

Szeged, 2017. 09. 27.

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Debrecen, 2017. 10. 03.