Short thesis for the degree of doctor of philosophy (PhD)

Characterization and application of Colorado potato beetle α-amylase

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Debrecen, 2022

1. Introduction:

Nowadays there is a global problem that people are provided with an appropriate quantity of food of appropriate quality. The reduction of agricultural damages is one of the solutions, for example reduce the attack by pests. There are several ways of defense, chemical or biological. Some of the herbivorous insects eat the crops of the plants, while there are leaf pests, such as the Colorado potato beetle (CPB). The potato beetle is highly adaptable as a food source and from the point of view of climate and insecticides, and there are few natural enemies in Europe, so biological protection proves to be ineffective against it, therefore its investigation has salient importance. One of the ways to control the CPB to inhibit the main digestive enzyme, the α-amylase. This control method complements and combines the plant protection strategies used until now. Many compounds found in nature have been proven to be α-amylase inhibitors, so we can use compounds that are selective, found in nature and thus not harmful for nature. In order to find suitable inhibitors, it is first of all essential to explore the active center structure of CPB α-amylase (LDAmy), which was my goal during my doctoral work.

The transfer activity of amylases is usually limited, but there are several examples in the literature that it can be increased by mutation. Preliminary studies showed that the wild type of LDAmy is also able to catalyze the transglycosylation reaction. To achieve the maximum activity in the direction of synthesis I examined the effect of pH and organic solvent concentration, since these parameters can significantly influence the functioning of the enzyme.

2. Materials and methods:

2. 1. Preparation of the CPB gut extract:

CPB larvae and adults were collected by the staff of the Plant Protection Institute of the Faculty of Agriculture, Food and Environmental Management of the University of Debrecen. The digestive organ of the insect was prepared and then placed in 250 and 500 µL of physiological saline solution (0.9% NaCl). The solution containing the intestinal extract was then vortexed for 30 seconds and then centrifuged for five minutes at 1000 g at 5 °C. The supernatant was separated and stored at -20 and -80 °C until the tests.

2.2. Enzyme activity measurement of the gut extract:

The activity of the carbohydrate-degrading enzymes (α-amylase, α-and β-glucosidase) of the insect gut extract was measured with 2-chloro-4-nitrophenyl-4-O-β-D-galactopyranosylmaltoside ($GalG_2CNP$), p-nitrophenyl-α - and β-D-glucopyranoside substrates by a Jasco V-550 spectrophotometer (Figure 1).

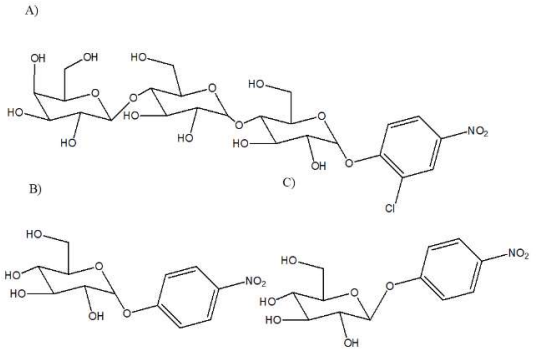

Figure 1: Structure of the substrates used to measure the enzyme activity of the insect gut extract: A) $GalG_2CNP$ B) p-nitrophenyl-α-D-glucopyranoside C) p-nitrophenyl-β-D-glucopyranoside.

The activity measurements were performed in a buffer (pH6) containing 50 mM 2-(N-morpholino)-ethanesulfonic acid (MES), 5 mM calcium acetate, 51.5 mM sodium chloride and 152 mM sodium azide at 37 °C. The reaction mixture contained 5 mM substrate dissolved in 200 µL MES buffer, 290 µL MES buffer and 10 µL insect gut extract. The change in absorbance was followed for 3 minutes at the absorption maximum of p-nitrophenol (400 nm).

2.3. Determination of action pattern of the gut extract:

During the examination of the action pattern of LDAmy the separation of the enzymatic products ~~produced~~ was carried out with an Agilent 1260 infinity II (quaternary pump, automatic injector, diode array detector) HPLC system, which an ESA Corona CAD detector was also attached. The reaction mixtures contained 1 mg of substrate in 1 mL of buffer. The reaction was started by adding 5 µL of gut extract. The separation was performed on an Accucore-aQ (150x2.1 mm, 2.6 µm, Thermo) column with a Security Guard Cartridge C18 (4x3.0 mm) column, the flow rate was 0.4 mL/min and the injection volume was 5 µL. The products from $CNPG_n$ substrates were separated by isocratic elution with a mixture of ACN:water 7:93 (V/V)%, while gradient elution was used to separate the hydrolysis products of $BnlG_nPNP$. The detection wavelengths were 200 and 302 nm.

2. 4. Purification of LDAmy enzyme:

The separation of LDAmy from the gut extract was carried out using affinity chromatography. A 5 mL portion of the extract in physiological salt solution was concentrated with a 30K ultrafilter at 5 °C and at 10,000 g, and a 200 µl portion of this sample was injected onto the crosslinked starch gel stationary phase. During the separation 1 mL fractions were collected. The full protein concentration in the fractions were monitored by spectrophotometry at 280 nm

wavelength. The α-amylase activity of the fractions was determined in the same way as the method used for the examination of the gut extract. The application buffer was 1 M acetate buffer, which contains 1 M ammonium sulfate (pH 5.2), as elution buffer I used 1 M acetate buffer (pH 5.2), which contained 10 mg/mL maltose. The purity of LDAmy was checked using SDS-PAGE.

2.5. Examination of the LDAmy synthesis reaction:

The synthesis reaction of LDAmy was examined by CNP-β-D-glucopyranoside, PNP-β-D-galactopyranoside, PNP-β-D-mannopyranoside, PNP-β-D-glucosaminide and PNP-β-D-xylopyranoside, as acceptors in the presence of glucose, maltose, maltotriose and a mixture of maltooligosaccharide as well as water-soluble starch as donors. The synthesis reaction was followed by HPLC applying the method used to examine the action pattern of the purified enzyme. To examine the donor molecules, I used 1% starch solution, 10 mM glucose, 10 and 25 mM maltose, 25 mM maltotriose or 10 mg/mL G5-G7 mixture dissolved in azide-free MES buffer. In each case, I started the reaction by adding 1 µL of 18 µM LDAmy solution. To determine the pH optimum of the transglycosylation reaction an azide free MES solution was used, the pH of which was set to 4, 5, 6, 7 and 8. Reaction mixtures with a volume of 1 mL contained 10 mg/mL G5-G7 mixture and 10 mM CNPG. I prepared 1 mL samples containing 10 mg/mL G5-G7 mixture and 10 mM CNPG donor in azide free MES buffer (pH6) and acetonitrile (ACN) solutions in order to investigate the effect of organic solvent on the synthesis reaction. The "preparative" separation of the products formed in the reaction mixture containing PNP-β-D-mannopyranoside was carried out on a Supelcosil LC-18 column (250*4.6mm, 5µm, Supelco) with a flow rate of 1.5 mL/min. The temperature of the column thermostat was 40 ◦C, the composition of the eluent was ACN:water 10:90

(V/V)%, and the injected volume was 25 µL. The effect of the quality of the non-reducing end protecting group on the transglycosylation reaction was investigated on BnlG7PNP and ethylidene-4-nitrophenyl-α-D-maltoheptaoside (Et-4-G7-NP, SORACHIM) substrates (Figure 2).

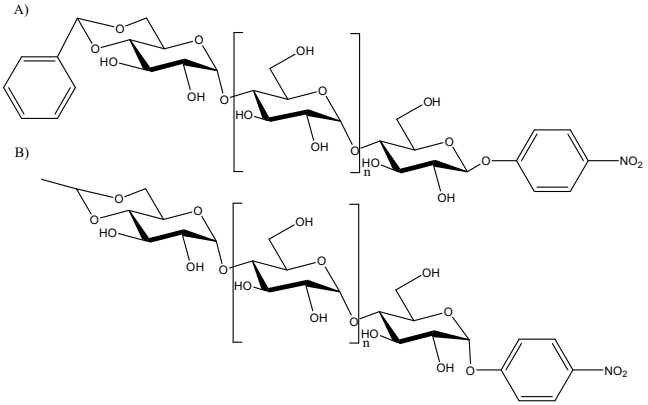

Figure 2: Structure of BnlG_7PNP (A) and Et-4-G7-NP (B) substrates

2.6. Determination of the action pattern of LDAmy by HPLC:

I performed the examination of the action pattern of the purified LDAmy on $CNPG_n$ (n=4-8) substrates. Reaction mixture contained ~100 µM substrate in 1 mL azide free MES buffer. The enzyme reaction was started with 1 µL of 18 µM LDAmy solution. The conditions of RP-HPLC method used for the tests was the following: Venusil AQ_C18 (150*4.6mm, 3 µm, Agela) column; 0.6 mL/min flow rate; eluent composition: ACN: Water 18:82 (V/V)%; injected volume: 5 µL; detection wavelength: 302 nm; measurement time: 20 minutes.

3. New scientific results

3.1. I proved that the carbohydrate-digesting enzymes of the potato beetle gut extract are α-amylase, α-glucosidase, and β-glucosidase.

I confirmed the presence of enzymes in the insect gut extract, which break down complex sugars formed from glucose units. The spectrophotometric measurements were carried out with specific substrates using conditions optimized for the α-amylase substrate (Figure 3).

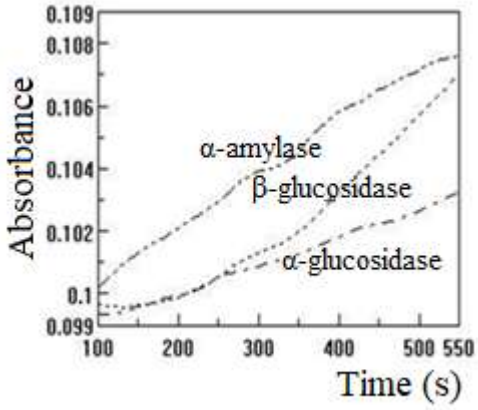

Figure 3: Examination of the α-amylase and α- and β-glucosidase activity of the midgut extract of the CPB

3.2. I investigated the carbohydrate breakdown of CPB gut extract on chromophore-containing maltooligosaccharide substrates. I found that the three enzymes have approximately the same reaction rate for the individual substrates and the products formed from them.

I determined the action pattern of the gut extract and compared it with the action pattern of α-amylases from other organisms. I performed the action pattern determinations on $CNPG_7$ and $BnlG_nPNP$ substrates, which contained n=4-8

glucose units. Since $CNPG_7$ is also a substrate for α-glucosidase, it consecutively breaks down the starting substrate and the products released by amylase from the non-reducing end. In the case of $BnlG_7PNP$, the hydrolysis products from the reducing end are substrates of α-glucosidase, but the primary hydrolysis products are exclusively products of α-amylase. I considered the activity of three enzymes to prepare the kinetic model. In the case of substrates with a smaller degree of polymerization (DP), the bond cleavage frequency values of LDAmy and PPA differed significantly from each other, so the active centers of the two enzymes should be different. During analysis of enzymatic reaction on BnlG8PNP, products could be detected, which indicated that, in addition to the hydrolysis, a reaction in the direction of synthesis also took place.

3.3. I successfully purified LDAmy from the gut extract using affinity chromatography on a starch gel stationary phase.

I prepared the affinity stationary phase by cross-linking of water-soluble starch. I used maltose-containing buffer for the elution, which binds competitively to the insect α-amylase enzyme. The purity of LDAmy was verified by SDS-PAGE and by measuring the spectrophotometric activity of carbohydrate-hydrolysing enzymes.

3.4. I found that LDAmy has transglycosylation activity and investigated the effect of the conditions on the transfer reaction.

I found that maltose can function as a donor molecule in the reaction, as well as maltooligosaccharides with a larger DP, with the exception of maltotriose, which inhibits the formation of transfer products. The pH optimum of the transglycosylation reaction was pH=6, at more acidic pH values the product ratio changed and there were a minimal decrease in the amount of all

transglycosylated products. The amount of products decreased drastically in the neutral and alkaline range. The presence of the organic solvent significantly affects the product ratio in the transglycosylation reaction, 20% ACN provides the highest synthesis/hydrolysis ratio. Transition metal ions have only a minor effect on the reaction. I observed the formation of products using only CNP-β-D-glucopyranoside and PNP-β-D-xylopyranoside, as acceptors. The quality of non-reducing end protecting group does not affect the ratio and quantity of the products.

3.5. I determined the action pattern of LDAmy by HPLC analysis using chromophore-containing maltooligosaccharide glycosides of DP 4-8.

The hydrolysis reaction was followed by the change in the area of the peaks obtained during the HPLC separation of the reducing end products of CNP maltooligomers. I determined the kinetic constants based on a hydrolysis model by software fitting of the kinetic curves (Figure 4).

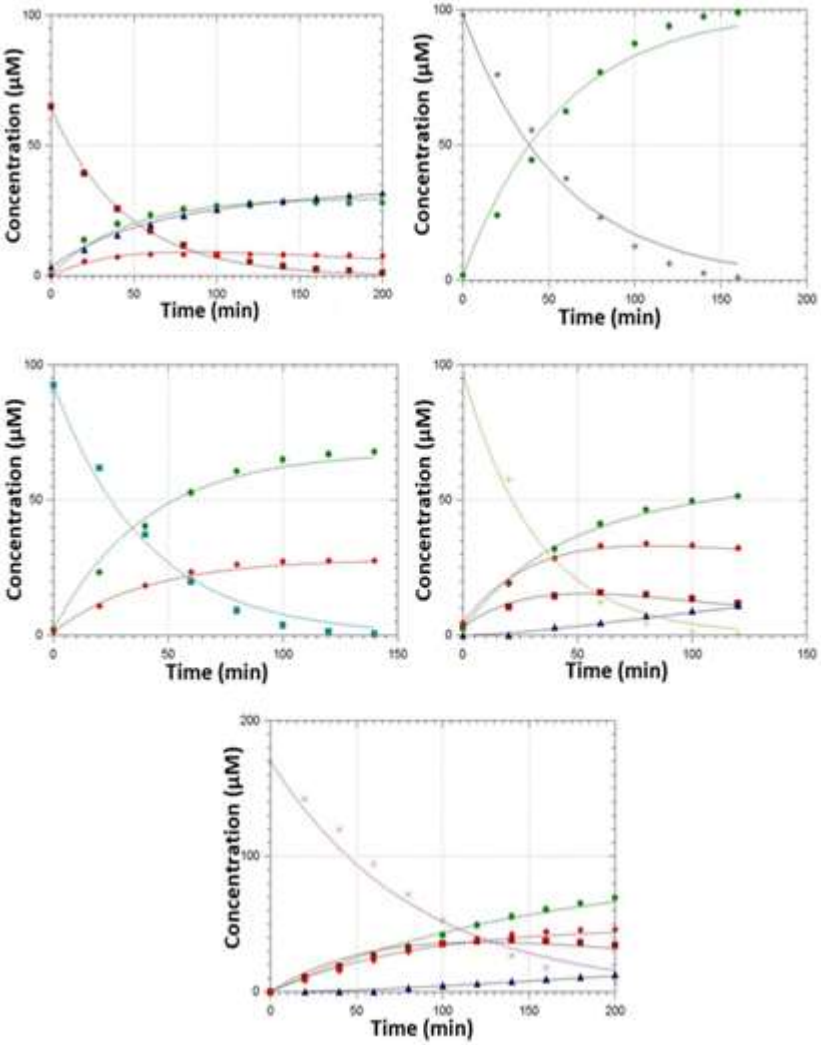

Figure 4: Examination of the action pattern of CNP maltooligomers. Changes in the concentration of starting substrate and products over time and the fitted kinetic curves ($CNPG_8$: X, $CNPG_7$: +, $CNPG_6$: ■, $CNPG_5$: *, $CNPG_4$: ■, $CNPG_3$: ♦, $CNPG_2$: ●, CNPG: ▲).

3.6. I determined the value of the bond cleavage frequencies. I created the subsite model of the LDAmy and compared it with the subsite map of α-amylases from other organisms.

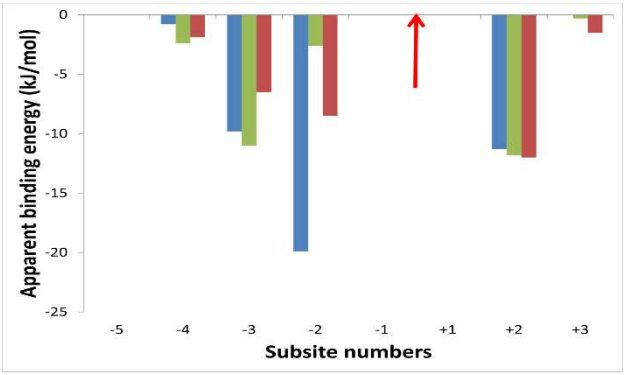

Figure 5: Subsite map of LDAmy (green), HSA (blue), and PPA (red). The numbers are the values of the apparent binding energies of LDAmy, the red arrow indicates the site of hydrolysis.

Based on the comparison of the LDAmy, PPA and HSA subsite maps, while the number of subsites was the same, significant energy differences can be observed for the glycon binding subsites (Figure 5). These differences are due to differences in the amino acid sequence of the three enzymes. I performed a sequence comparison for the enzymes LDAmy, HSA, PPA and TMA. The energy differences visible on the subsite map can always be connected to the significant polarity differences appearing in the sequences. LDAmy contains an OH-containing threonine at the subsite -4, where HSA has a negatively charged aspartic acid side chain. At the -3 glycon binding site of HSA contains a serine side chain, where LDAmy has a non-polar valine, similar to PPA and TMA. The biggest difference in the binding energy is at the subsite -2, where HSA contains a basic histidine and LDAmy contains a negatively charged aspartic acid side chain.

4. Possibilities of utilizing the results:

The obtained results can help in the search for natural inhibitors, as well as in the design and development of synthetic inhibitors. Recognising the transfer activity of insect α-amylase, as well as the discovery of the sequence differences, creates opportunity to introduce higher transferase activity to other α-amylases.

Publications

Lectures on conferences:

1. Hámori Csaba, Rovar eredetű α-amiláz tisztítása és jellemzése. XXIII. Tavaszi Szél Konferencia, Budapest 2020. ISBN: 978-615-5586-70-5
2. Hámori Csaba, Szénhidráton ható enzimek aktivitásmérése HPLC módszerrel. XXII. Tavaszi Szél Konferencia, Budapest 2019. ISBN 978-615-5586-42-2
3. Hámori Csaba, A kolorádóbogár szénhidrát emésztésének vizsgálata. PEME XVII. PhD – Konferencia Budapest 2018.

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1. C. Hámori, L. Kandra, G. Gyémánt, α-Amylase as the main enzyme in the digestive tract of a leaf-consuming insect Colorado potato beetle (*Leptinotarsa decemlineata,* Say). 7th Symposium on the Alpha-Amylase Family, Smolenice, 2019.
2. K. Szabó, C. Hámori, G. Gyémánt, Study on the aggregation tendency of tannins – or are tannins specific α-amylase inhibitors? 7th Symposium on the Alpha-Amylase Family, Smolenice, 2019.

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Registry number: DEENK/432/2022.PL
Subject: PhD Publication List

Candidate: Csaba Hámori
Doctoral School: Doctoral School of Chemistry
MTMT ID: 10069158

List of publications related to the dissertation

Foreign language scientific articles in international journals (3)

1. **Hámori, C.**, Kandra, L., Gyémánt, G.: LDAmy, an α-amylase from Colorado potato beetle (Leptinotarsa decemlineata) with transglycosylation activity.
   Biocatal. Biotransform. [Epub ahead of print], 2022. ISSN: 1024-2422.
   DOI: http://dx.doi.org/10.1080/10242422.2022.2050707
   IF: 2.325 (2021)

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   Int. J. Biol. Macromol. 168, 350-355, 2021. ISSN: 0141-8130.
   DOI: http://dx.doi.org/10.1016/j.ijbiomac.2020.12.071
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3. Szilágyi, E., **Hámori, C.**, Bíróné Molnár, P., Kandra, L., Gálné Remenyik, J., Gyémánt, G.: Cooperation of enzymes involved in carbohydrate digestion of Colorado potato beetle (Leptinotarsa decemlineata, Say).
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   DOI: http://dx.doi.org/10.1017/S0007485319000099
   IF: 1.814

List of other publications

Foreign language scientific articles in international journals (3)

4. Jószai, I., Balogh, C. A., **Hámori, C.**, Molnár, D., Forgács, V., Rubleczky, B., Trencsényi, G.: Determination of Total Radiochemical Purity of [18F]FDG and [18F]FET by High-Performance Liquid Chromatography Avoiding TLC Method.
   Chromatographia. 85, 469-479, 2022. ISSN: 0009-5893.
   DOI: http://dx.doi.org/10.1007/s10337-022-04155-x
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5. Nagy-Szabó, K. A., **Hámori, C.**, Gyémánt, G.: Gallotannins are Non-Specific Inhibitors of α-Amylase: Aggregates are the Active Species taking part in Inhibition.
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DOI: https://doi.org/10.1111/cbdd.13787
IF: 2.873

6. Király, A., **Hámori, C.**, Gyémánt, G., Kövér, K. E., Pócsi, I., Leiter, É.: Characterization of gfdB, putatively encoding a glycerol 3-phosphate dehydrogenase in Aspergillus nidulans.
Fungal Biology. 124 (5), 352-360, 2020. ISSN: 1878-6146.
DOI: http://dx.doi.org/10.1016/j.funbio.2019.09.011
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Other journal articles (1)

7. Jószai, I., **Hámori, C.**, Balogh, B., Pótári, N., Mikecz, P.: Simultaneous analysis of FDG, CIDG and radiochemical components in [18F]FDG preparation by a new NP-HPLC method (P-392).
J. Labelled Comp. Rad. 56 (Suppl.), S-479, 2013. ISSN: 0362-4803.

Hungarian abstracts (3)

8. Király, A., **Hámori, C.**, Gyémánt, G., Kövér, K. E., Pócsi, I., Leiter, É.: A glicerin-3-foszfát dehidrogenáz (gfdB) gén szerepe az Aspergillus nidulans és Aspergillus glaucus oxidatív stresszválaszában.
In: Biotechnológia a Debreceni Egyetemen Tudományos szimpózium: Program és összefoglalók, Debreceni Egyetem Természettudományi Kar, Debrecen, 20, 2019.

9. Balogh, C. A., **Hámori, C.**, Jószai, I.: Az O-(2-[18F]fluoroetil)-L-tirozin tartalmú PET diagnosztikum radiokémiai tisztaságvizsgálata új folyadékkromatográfiás eljárással.
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10. **Hámori, C.**, Jószai, I.: A [18F]FDG gyógyszerkészítmény radiokémiai tisztaságának vizsgálata hidrofil kölcsönhatási folyadékkromatográfia (HILIC) segítségével.
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Foreign language abstracts (4)

11. Király, A., Molnár, A., Bodnár, V., **Hámori, C.**, Gyémánt, G., Kövér, K. E., Leiter, É., Pócsi, I.: Glycerol-3-phosphate dehydrogenase GfdB in the oxidative stress defense of Aspergillus nidulans.
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12. Jószai, I., **Hámori, C.**: HILIC-UPLC for fast determination of radiochemical purity of [18F]FDG. *J. Label. Compd. Radiopharm. 60* (1), S478, 2017. ISSN: 0362-4803. DOI: http://dx.doi.org/10.1002/jlcr.3506

13. **Hámori, C.**, Balogh, C. A., Jószai, I.: Significance of ph control in determination of radiochemical purity of f-18 labeled tracers using liquid chromatography. *Nuc. Med. Rev. 20* (2), 111, 2017. ISSN: 1506-9680.

14. Jószai, I., **Hámori, C.**, Balogh, B., Pótári, N., Mikecz, P.: Simultaneous determination of radiochemical and chemical purity of [18F] FDG by new normal phase HPLC method (P4). *Nucl. Med. Rev. Cent. E. Eur. 16* (Suppl.), A21, 2013. ISSN: 1506-9680.

Total IF of journals (all publications): 20,349
Total IF of journals (publications related to the dissertation): 12,164

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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