PH.D. THESIS

THE STUDY OF LACTOSE UTILISATION IN *PENICILLIUM CHRYSOGENUM*

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INTRODUCTION

Industrial production of penicillin is carried out by *Penicillium chrysogenum*. Penicillin biosynthesis is subject to carbon source regulation. Glucose, fructose, galactose and other easily utilised carbon sources reduce penicillin titer while the titer on lactose is high. Glucose and other easily utilisable carbon sources have a negative effect on the expression of penicillin biosynthetic genes, but lactose does not have such an effect. Lactose was therefore previously used as carbon source in penicillin production. Nowadays, this problem has been partially overcome by feeding subrepressing doses of glucose. Since in general the fungus grows better on glucose than on lactose, the production of penicillin appears to be favoured by suboptimal growth conditions (Brakhage, A. A., 1998. Molecular regulation of β-lactam biosynthesis in filamentous fungi. Microbiol. Mol. Biol. Rev. **62,** 547-585.). Many studies have been reported on the repressing effect of glucose and its role in penicillin biosynthesis regulation, however lactose utilisation and its regulation have not been thoroughly investigated. Elucidating of these questions could help to develop high antibiotic producer industrial strains and design optimal growth conditions.

In contrast to other bacteria, yeasts and filamentous fungi in its natural habitat *Penicillium chrysogenum* does not occur on media containing lactose as carbon source. Therefore comparing of their lactose utilisation mechanism can reveal new utilisation pathway and regulation system. Many studies reported that the mechanisms are varied, and sometimes exist in a species in parallel or it is specific for a species.

Since the mechanism of lactose utilisation in *Penicillium chrysogenum* had not been investigated, we found it worth studying. An industrial penicillin producer strain of *Penicillium chrysogenum* was used in our experiments. Our work mainly focused on utilisation of lactose as carbon source and its regulation forming the base of a wide physiological and genetic study.

The “key step” of lactose utilisation is the hydrolysis of the molecule to glucose and galactose. The reaction is catalysed by a glycohydrolase enzyme, the β-galactosidase. This enzyme was isolated from animals, plants and microorganisms as well. The β-galactosidase in bacteria in yeasts and in filamentous fungi is generally intracellular and may be induced by lactose, but in several species it is extracellular.

It is well-known that glucose and other easily metabolisable sugars inhibit the synthesis of β-galactosidase, causing repression of the enzyme biosynthesis. The enzyme and the regulation of the enzyme synthesis were most thoroughly studied and understood at the molecular level in *E. coli*.

For a long time only the cAMP-dependent mechanism of carbon catabolite repression in *E. coli* was known. It is well demonstrated that in the presence of glucose the low activity of the catabolite sensitive enzyme like β-galactosidase correlates with low cAMP concentration. Later two cAMP independent catabolite repression mechanism were revealed in bacteria. One mechanism found in *E. coli* and another in *Bacillus subtilis* (Saier, M. H. Jr. 1996. Cyclic AMP-independent catabolite repression in bacteria. FEMS Microbiol. Lett. **138:** 97-103.).

The mechanism of glucose repression in yeasts and other fungi is entirely different from that of bacteria. In glucose repression cAMP is not involved or its role is different (Ronne, H. 1995. Glucose repression in fungi. TIG. **11:** 12-17.). However our knowledge in this field is quite limited. In carbon repression mechanism zinc-finger type repressor proteins have a significant role, like the MIG in yeasts, and the CREA in *Aspergillus*. In filamentous fungi the mechanism of carbon repression of antibiotic production is well studied. Carbon source acts at several points of the penicillin biosynthesis and it seems that the mechanism of carbon repression of antibiotic synthesis is similar to the mechanism of carbon repression of carbohydrate utilisation. Investigation of the role and regulation of β-galactosidase could result more detailed comparison of the two mechanisms. Enzymes with β-galactosidase activity were purified from different fungi and characterised. These enzymes are mainly extracellular and have higher temperature and lower pH optimum comparing them to β-galactosidases from other microbial sources. These advantages of the mould β-galactosidase are dominating at the choice of enzyme for industrial production and application. Although many microbial β-galactosidases have been investigated for this purpose, very few studies have been reported on the mechanism and regulation of enzyme production.

Our work mainly focused on the physiology of the antibiotic producer industrial strain of *Penicillium chrysogenum*. We investigated the carbon repression in this filamentous fungus by studying the intracellular production of β-galactosidase and the mechanism of induction/repression of the enzyme. We examined the kinetics of enzyme appearance

after induction, the types of compounds and other factors, such as cAMP, which might affect the carbon repression mechanism. We determined properties of the enzyme and its genetic background. Comparing these results to others we want to conclude the mechanism of carbon repression.

Aim of the study:

1. Is lactose hydrolysis cell bound or extracellular? Is the β-galactosidase responsible for the hydrolysis intracellular or extracellular? How does the enzyme activity change during growth?
2. How do different carbon sources influence the growth of the fungi? Which carbon sources support β-galactosidase production? May the enzyme synthesis be induced? How effective the enzyme is in lactose utilisation?
3. Is there any correlation between β-galactosidase activity and intracellular cAMP level? Is it possible that cAMP has a role in the regulation of enzyme production? How does caffeine as a phosphodiesterase inhibitor affect carbon source regulation? Does the cAMP analog 6-*N*-2-*O*-dibutiryl-cyclic-AMP (dBcAMP) influence the β-galactosidase production?
4. What properties does the purified enzyme have? What similarities does the enzyme show to other microbial β-galactosidases?
5. How many genes encode the β-galactosidase enzyme?
6. Are there any other proteins involved in the regulation of the enzyme function?

MATERIALS AND METHODS

The liquid shaken culture of the industrial strain of *Penicillium chrysogenum* NCAIM 00237 was used in our experiments. β-galactosidase, hexosaminidase, α-glucosidase, β-glucosidase activities and glucose concentration of the broth were determined spectrophotometrically with rate-assay method. Lactose concentration and the concentration of intracellular nucleotides were determined by HPLC analysis. The ammonia concentration was measured in the culture filtrates by an ammonia sensitive electrode set. The intracellular β-galactosidase enzyme was purified by procedures including precipitation with ammonium sulphate, DEAE-Sephadex ion-exchange chromatography, affinity chromatography, and chromatofocusing. The purification steps, the molecular weight of the enzyme subunits, and the protease activity were monitored and determined by SDS- and native PAGE. The molecular weight of the native enzyme was determined on Sephacryl S-300 column. For determination of N-terminal amino acid sequences proteins were blotted to polyvinildiaen-fluoride membrane by Semi-Dry System (Single Transfer) (Millipore Corporation). N-terminals of the proteins were sequenced by Edman degradation. Basic molecular biology methods (isolation of chromosomal DNA; agarose gel electrophoresis; amplification of DNA fragments by polymerase chain reaction; isolation of plasmid from *E. coli*; Southern hybridisation) were carried out by the protocols of J. Sambrook, E. F. Fritsch, T. Maniatis [1989. Molecular Cloning, A Laboratory Manual (Second Edition), Cold Spring Harbor Laboratory Press handbook]. PCR products were cloned by Zero Blunt™ PCR Cloning Kit (Invitrogen). The DNA fragments were sequenced by ABI 373 automated sequencer (Applied Biosystems). Immunological detection of hybridisation was carried out by DIG Nucleic Acid Detection Kit (Boehringer Mannheim).

RESULTS

Physiological experiments

- Our experiments showed that β-galactosidase activity in *Penicillium chrysogenum* remained cell bound. The low activity in the culture fluid was probably due to cell lysis at later stages of growth. Intracellular β-galactosidase activity reached the maximum level at 60- to 70-h period of the growth in complex liquid medium containing 2 % lactose as a sole carbon source.
- Glucose, sucrose and suprisingly galactose were the best, easily metabolised carbon sources giving relatively fast growth. Cultures reached the highest biomass by the 48th hour of the growth, then the growth stopped, and cell lysis started. Glycerol proved to be a fairly good carbon source but it was metabolised slower. Lactose was a rather poor carbon source, giving much less biomass. In the lactose grown culture high ammonia production was observed until β-galactosidase activity reached a relatively high level, which showed that peptone was a better energy source than lactose.
- In *Penicillium chrysogenum* among the carbon sources tested the production of β-galactosidase was high only on lactose. In glucose, sucrose, galactose and glycerol grown cultures practically no enzyme activity was detected during exponential growth. In our strain the enzyme was induced only by lactose. This is in contrast of other fungi (e.g. *Aspergillus nidulans*, *Neurospora crassa*) and yeasts where lactose and galactose are also effective inducers of β-galactosidase.
- When glucose, galactose, sucrose or glycerol were added to lactose grown cultures the enzyme level declined until the exhaustion of these carbon sources. This means that glucose, galactose, sucrose and glycerol repressed the β-galactosidase biosynthesis. Repressing effect of glucose was concentration dependent similarly to the repression of penicillin biosynthesis.
- In glucose grown complex cultures the correlation between the cessation of growth, depletion of glucose and the appearance of the low level of β-galactosidase suggests that in *Penicillium chrysogenum* there was an uninduced, derepressed synthesis of the enzyme. Derepression was weak but significant.
- Our results showed relatively high cAMP level in lactose and low level in glucose grown cells. In glycerol grown cultures there were no detectable amounts of cAMP,

however on sucrose as high cAMP level was measured as in lactose grown cells. Because sucrose repressed the β-galactosidase synthesis, and increased growth rate like glucose these results suggested that there was no correlation between cAMP level and carbon repression or carbon starvation directly in *Penicillium chrysogenum* like in other filamentous fungus.

Investigating the effect of caffeine we found that cAMP did not influence the β-galactosidase synthesis directly. Assuming that the main effect of caffeine is to increase cAMP level, cAMP had no role in carbon repression of β-galactosidase synthesis but caffeine strongly antagonised glucose consumption. It is quite possible that in our case the reduced glucose consumption was due to the inhibition of glucose uptake.

The cAMP analog dBcAMP had no effect on growth and β-galactosidase activity. The reasons for this were not further investigated.

- These results comparing to knowledge of carbon regulation of penicillin biosynthesis also suggest that a common regulatory mechanism is involved in carbon regulation of both sugar utilisation and penicillin biosynthesis.

Enzymology

- Intracellular β-galactosidase from *Penicillium chrysogenum* was purified by procedures including precipitation with ammonium sulphate, ion-exchange chromatography on DEAE-Sephadex, affinity chromatography, and chromatofocusing. These steps resulted in a purification of 66-fold, and a yield of 8 %.
- Some enzyme characteristics were determined using *o*-nitrophenyl-β-D-galactopyranoside as substrate. The purified enzyme exhibited a broad pH optimum of pH 4-5 where it exerted more than 95 % of its maximum activity. The optimum temperature range of β-galactosidase activity was 27-37 ^{o}C. The isoelectric point of the protein proved to be 4.6. The calculated kinetic constants of the enzyme are K_m=1.81 mM, V_{max}=40 nkat/mg using ONPG, K_m=27,02 mM, V_{max}=9,2 nkat/mg using lactose as substrate. The K_m values are very similar to the data obtained for *Aspergillus nidulans* and *Aspergillus oryzae*. Investigating of the substrate specificity of the enzyme we found that the purified enzyme hydrolysed ONPG, PNP-GlcNAc and lactose but had no α-glucosidase and β-glucosidase activities.

Substrate specificity of the enzyme showed that it had hexosaminidase activity. Enzyme properties of the purified β-galactosidase were compared to the β-*N*-acetylhexosaminidase, which was also characterised in *Penicillium chrysogenum* (Pusztehelyi, T. 1997. Öregedő *Penicillium chrysogenum* tenyészetek élettani vizsgálata. Ph.D. értekezés. Kossuth Lajos Tudományegyetem, Debrecen).

The determined kinetic constants showed that β-galactosidase had quite high hexosaminidase activity, but it was lower than this activity of β-*N*-acetylhexosaminidase. An important difference was that the purified β-*N*-acetylhexosaminidase hydrolysed neither lactose nor ONPG the best synthetic substrate of β-galactosidases. Another difference was observed in temperature optimum of the two enzymes. The β-galactosidase is a quite large, tetrameric protein but β-*N*-acetylhexosaminidase is a dimer in *Penicillium chrysogenum*. However the molecular weights of their subunits were very similar. We determined the N-terminal amino acid sequence of the subunit of β-galactosidase and it was found that this sequence is identical to the 99-113. amino acid residues of the previously cloned and sequenced β-N-acetylhexosaminidase of *Penicillium chrysogenum* (Diez, B.; Barredo, J. L. 1998. The beta-N-acetylhexosaminidase encoding gene from *Penicillium chrysogenum*. Adva. Antibioticos 59-61.). This protein has an 18 amino acid long signal sequence. The 97-98. amino acids are KR which is the cleavage site of the Kex2 endopeptidase in yeasts.

- During the purification of the β-galactosidase from *Penicillium chrysogenum* two other proteins co-purified with it. We also determined their N-terminal sequences. One (83 kDa) of them has an almost identical amino terminus (10 out of 11 residues are identical) to the amino-terminal sequence of the mature dipeptidyl-peptidase of *Aspergillus fumigatus* generated by the removal of the signal sequence (Beauvais, A.; Monod, M.; Svab, J.; Kobayashi, H.; Diauqin, M.; Hovanessian, A. G.; Latge, J. P. 1997. Biochemical and antigenic characterization of a new dipeptidyl-peptidase isolated from *Aspergillus fumigatus*. J. Biol. Chem. **272(10):** 6238-6244.). The other (78 kDa) protein shows no significant homology to any known protein. This latter protein however has a Ca^{2+} dependent proteolytic activity. These proteins might be involved in the proteolytic processing of β-galactosidase.

Genetic experiments

- Genetic analysis of β-galactosidase and β-*N*-acetylhexosaminidase from *Penicillium chrysogenum* showed that the constructed oligonucleotide for N-terminal sequence of β-galactosidase was hybridised to one fragment of digested genomic DNA of *Penicillium chrysogenum*. This 45 mer length oligonucleitide was identical to the 1618-1662 fragment of the 5'-end of β-*N*-acetylhexosaminidase gene. As we found one signal in hybridisation and the sequence of the 628 bp length DNA fragment amplified by the designed primers was absolutely identical to the 1618-2306 fragment of β-*N*-acetylhexosaminidase gene containing the hybridised fragment as well we conclude that one gene encodes the subunits of β-galactosidase and β-*N*-acetylhexosaminidase in *Penicillium chrysogenum*.
- Based on these results we propose that in our industrial penicillin producer strain lactose is hydrolysed by a glycohydrolase with broad substrate specificity that has low affinity for lactose. This explains why lactose is a „non-easily metabolised" carbon source. Maturation/activation of the protein takes place trough limited post-translational proteolysis. The role of the co-purifying proteins in this process is an intriguing question.

PUBLICATIONS

List of publications related to the thesis

Z. Nagy, T. Kiss, A. Szentirmai, S. Biró: β-Galactosidase of *Penicillium chrsogenum:* Production, Purification, and Characterization of the Enzyme. Protein Expression and Purification, 21(1), 24-29 (2001). Impf.: 1.569

Z. Nagy, Zs. Keresztessy, A. Szentirmai, S. Biró: Carbon source regulation of β-galactosidase in *Penicillium chrysogenum*. J. Basic Microbiol. 41(6), 351-362 (2001). Impf.: 0.613

Z. Nagy, A. Szentirmai, S. Biró: A multifunctional glycohydrolase is responsible for lactose utilisation in an industrial *Penicillium chrysogenum* strain. Under construction.

Lectures and posters

Nagy Z., Keresztessy Zs., Szentirmai A., Biró S.: Katabolit represszió vizsgálata *Penicillium chrysogenum*ban. A Magyar Mikrobiológiai Társaság 15. Nagygyűlése, Miskolc 1998.

Nagy Z., Szentirmai A., Biró S.: Purification and properties of β-galactosidase from *Penicillium chrysogenum*. 13th International Congress of the Hungarian Society for Microbiology, Budapest 1999.

Nagy Z., Szentirmai A., Biró S.: Carbon catabolite repression of β-galactosidase biosynthesis by *Penicillium chrysogenum*. A Magyar Mikrobiológiai Társaság 2000. évi Nagygyűlése, 2000.

Nagy Z., Szentirmai A., Biró S.: Laktóz metabolizmus *Penicillium chrysogenum*ban: mely enzim hidrolizálja a laktózt? A Magyar Mikrobiológiai Társaság 2001. évi Nagygyűlése, 2001.

Other publications

J. Kerékgyártó, **Z. Nagy**, Z. Szurmai: Synthesis of spacer-armed carbohydrate model compounds. Carbohydrate Research, 297, 107-115 (1997). Impf.: 1.606

Z. Nagy, A. Szentirmai, A. J. Clutterbuck, S. Biró: Isolating and mapping β-galactosidase negative mutants of *Aspergillus nidulans* and *Penicillium chrysogenum*. Acta Microbiologica et Immunologica Hungarica 46 (2-3), 342 (1999).

Nagy Z., A. J. Clutterbuck., Szentirmai A., Biró S.: *Aspergillus nidulans* β-galaktozidáz mutáns izolálása és térképezése, I. Magyar Mikológiai Konferencia, Budapest 1999.