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PENICILLIN G SECRETION BY INDUSTRIAL MYCELIA OF *PENICILLIUM CHRYSOGENUM*

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Wydalanie penicyliny G z przemysłowej grzybni *Penicillium Chrysogenum*

Streszczenie: W hodowlach przemysłowych penicylina G jest wydalana z komórek grzybni wysokowydajnych szczepów *Penicillium chrysogenum* w ilości 50–55 g w litrze podłoża. Efektywne wydalanie tak olbrzymiej ilość antybiotyku z komórek grzybni przemysłowej wymaga licznych dostosowań ultrastrukturalnych. Doniesienia literaturowe wskazują, że transportery ABC są całkowicie niewydajne w tym zakresie aktywności biosyntezy penicyliny G. Obecnie, inny mechanizm sekrecji antybiotyku z komórek grzybni szczepów przemysłowych powinien być brany pod uwagę, tj. proces egzocytozy. Ostatnie dwa enzymy na szlaku biosyntezy penicyliny G są zlokalizowane w peroksyzomach, gdzie antybiotyk jest gromadzony. Wyniki naszych wieloletnich badań wspierają hipotezę, że peksofagia (selektywne pochłanianie i degradacja dużych peroksysomów w wakuolach), a następnie egzocytoza, tj. pączkowania wakuoli i tworzenie wakuolarnych zbiorników oraz fuzja tych zbiorników z błoną cytoplazmatyczną są procesami, które mogą uczestniczyć w transportowaniu penicyliny G z komórek przemysłowej grzybni do podłoża fermentacyjnego.

Abstract: In industrial cultures, the extracellular penicillin G is secreted by the overproducing strains of *Penicillium chrysogenum* in amounts of 50–55 g per liter of the liquid fermentation medium. Such an abundant amount of the produced antibiotic requires a specially adopted ultra-structural organization to secrete the antibiotic from the industrial mycelium, because the traditional ABC-transporters are not efficient enough. Thus, a novel secretory mechanism, i.e. the process of exocytosis, should be now also taken into account. The last step in penicillin G biosynthesis is located in peroxisomes, in which the final product is subsequently entrapped. The results of our long-term experiments support the hypothesis that vacuolar pexophagy (phagy of peroxisomes) combined with exocytosis, i.e. vacuolar budding resulting in the formation of numerous vacuolar vesicles followed by their fusion with the plasma membrane, might be directly involved in penicillin G secretion from the industrial mycelium of *P. chrysogenum*.

Key words: exocytosis, *P. chrysogenum*, penicillin G, pexophagy
Słowa kluczowe: egzocytoza, *P. chrysogenum*, peksofagia, penicylina G

1. Introduction

The last two enzymes in penicillin G biosynthesis, i.e. phenylacetyl-CoA ligase and acyl-CoA:6-aminopenicillanic acid acyltransferase are located in peroxisomes called also microbodies. Secretion of penicillin G from the interior of peroxisomes first across the peroxisomal membrane and then through the plasma membrane of the high-penicillin-yielding mycelial cells of *P. chrysogenum* is at present poorly understood [1, 19]. Actually, there are no known transporters involved in the large-scale secretion of penicillin G from the industrial mycelia [8, 16, 17].

This article is designed to overview details concerned with the hypothesis that pexophagy and exocytosis might be immediately involved in the process of penicillin G secretion from the industrial mycelium of *P. chrysogenum* into the fermentation medium. Another goal was to discuss further ultra-structural organization of the industrial mycelium which is combined with the large-scale biosynthesis and secretion of penicillin G.

2. The pathway of penicillin G biosynthesis

β-Lactams have unusual chemical structures, like many other secondary metabolites [7]. All β-lactams are composed of a four-membered β-lactam ring closed by an amide bond. Penicillins contain a bicyclic penam nucleus formed by fused β-lactam and

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thiazolidine rings and an acyl side-chain bound to the amino group at C-6. The biosynthesis of penicillin G starts by the condensation of activated precursor amino acids L-α-aminoadipic acid, L-cysteine and L-valine to δ-L-α-aminoadipyl-L-cysteinyl-D-valine (ACV) tri-peptide formed by the ACV synthetase – encoded by the *pcbAB* gene. This tri-peptide is converted to isopenicillin N (IPN) by the action of IPN synthase – encoded by the *pcbC* gene. In this ring closure reaction the bi-cyclic penam nucleus is formed consisting of β-lactam and thiazolidine rings. In the last step, the acyl-CoA:6-aminopenicillanic acid acyltransferase encoded by the *penDE* gene synthesizes penicillin G by substitution of the L-α-aminoadipyl side chain of IPN by the phenylacetyl side chain [7].

3. Ultrastructural arrangements in penicillin G secretion by industrial strains

At the highest activity of penicillin G secretion, the industrial hyphae of *P. chrysogenum* is composed of apical regions, growing sub-apical cells, sub-apical metabolically active non-growing vacuolated cells, and late-apical degenerating highly vacuolated cells. At the high activity of penicillin G secretion about 75% of the mycelial cells were characterized as vacuolated cells [7]. In the sub-apical non-growing cells the widely extending endoplasmic reticulum is frequently placed at the periphery of the cytoplasm and around the vacuoles transforming into numerous peroxisomes from 0.1 μm up to 1.0 μm in diameter which are characteristically accompanied by the rough endoplasmic reticulum and polyribosomes. The abundant pexophagy of mature peroxisomes and exocytosis are important features characterizing the non-growing mycelia cells of the high-yielding strains.

4. The "assembly line – the machinery" of penicillin G biosynthesis

Penicillin G biosynthesis and secretion by *P. chrysogenum* is a compartmentalized process. The enzymes of the penicillin biosynthetic pathway are located in different cellular compartments. For this reason, the precursors of penicillin G biosynthesis and the biologically active end-product have to cross one or more membranes. The final enzyme step in penicillin G biosynthesis is mediated by acyl-CoA:6-aminopenicillanic acid acyltransferae which is located in peroxisomes. At the high activity, the "machinery" of penicillin G overproduction build a strictly adjusted structurally arranged "assembly line" consisting of peroxisome entrapped phenylacetyl-CoA ligase and acyl-CoA:6-aminopenicillanic acyltransferase. These enzymes are close co-located with the IPN synthase which is mainly accumulated in the cytosol between the peroxisome and the peroxisome surrounding polyribosomes that are abundantly accompanied by the endoplasmic reticulum [7]. The pH in the lumen of peroxisomes is around 7.5 and is in the same range as the cytosoloc pH. It corresponds to the highest activity of all key enzymes of the penicillin biosynthetic pathway which are inactive at pH values below 6.0 [18]. These findings are in accordance with the active status of the cytosolic ACV synthetase in penicillin G biosynthesis [18]. The purified enzyme has a sharp pH optimum about 7.5 [15] corresponding with the cytosolic pH of approximately 7.0–7.5.

The productive mycelial cells of the high-yielding strain exhibit numerous mature peroxisomes frequently located at the periphery of the cytoplasm and around the vacuoles. Such an arrangement may increase the peroxisome accompanied IPN synthase supplying efficacy in penicillin biosynthesis from the medium and from the cytosol as well as from the vacuolar pool. These discoveries are consistent with the reported positive correlation between the number of mature peroxisomes and penicillin G biosynthesis [3, 4, 10, 11]. Two models of peroxisome development have been documented, the *de novo* synthesis from the endoplasmic reticulum and multiplication by fission of pre-existing organelles [2, 10, 12, 13].

5. The ABC-transporters in penicillin G secretion

At present, secretion of penicillin G from the mycelium of the industrial strains to the fermentation medium is only poorly understood [19]. Penicillin G is secreted from the industrial mycelium in very large quantities. Since the last two steps in penicillin G biosynthesis are located in peroxisomes the antibiotic has to be transported from the peroxisomes to the culture medium. In spite of significant effort that has been made to explain the role of the ABC transporters in penicillin G secretion by the high-yielding strains, the lack of clear involvement of any of these transporters may indicate that secretion of this antibiotic does not proceed through the classical ABC pump, although this might be the case in the wild-type strains [9]. Therefore, at present, new secretory pathways are considered for penicillin G secretion by the industrial strains that were absent or inefficient in the low-yielding wild-type strains.

6. Pexophagy and exocytosis in penicillin G secretion by high-yielding strains

Pexophagy was detected mainly in sub-apical non-growing productive vacuolated cells and in late-apical degenerating highly vacuolated cells of the mycelium of the high-penicillin-producing strain *P. chrysogenum* PQ-96 [7]. Inside of vacuolar invaginations

abundant pexophagy of large and middle size peroxisomes is a characteristic feature at the period of intensive penicillin G secretion (Fig. 1). A dominant feature of the productive and late-apical hyphal cells of the industrial strain is the high cellular vacuolization and abundant budding of vacuoles, resulting in formation of numerous vacuolar vesicles of about 0.3 μm to 0.4 μm in diameter. The intensive vacuolar budding (Fig. 2) is characteristic during the period

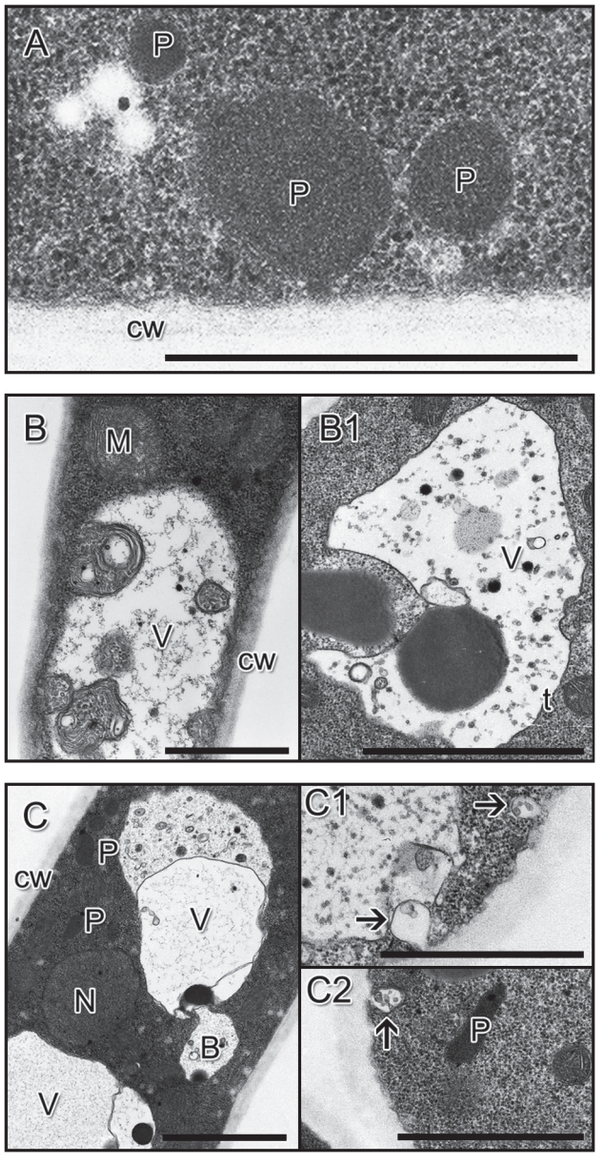

Fig. 1. *P. chrysogenum* PQ-96, 72 h culture, high-penicillin-producing strain, activity of penicillin G biosynthesis (U/ml): total yield at 72 h of cultivation – 8400, increase of yield between 48 h and 72 h of fermentation – 5800. (**A**) Penicillin G is produced in peroxisomes (P), which are frequently located in the peripheral cytoplasm. (**B**) Autophagy – vacuole (V) engulfed mitochondria (M) and (**B1**) abundant pexophagy are characteristic ultra-structural features of the vacuolated mycelial cells at the highest activity of penicillin G secretion from the mycelium into the fermentation medium. In the tonoplast (t) surrounded vacuole numerous organelle debris including the damaged peroxisomal matrix (black spots) can be observed. (**C**) At the cell wall (cw) located peroxisomes (P) and vacuole (V) engulfed peroxisomes are associated with the process of vacuolar budding (B). (**C1** and **C2**) The exocytosis (fusion of vacuolar vesicles with plasma membrane – arrows) is shown. Scale bar = 1 μm.

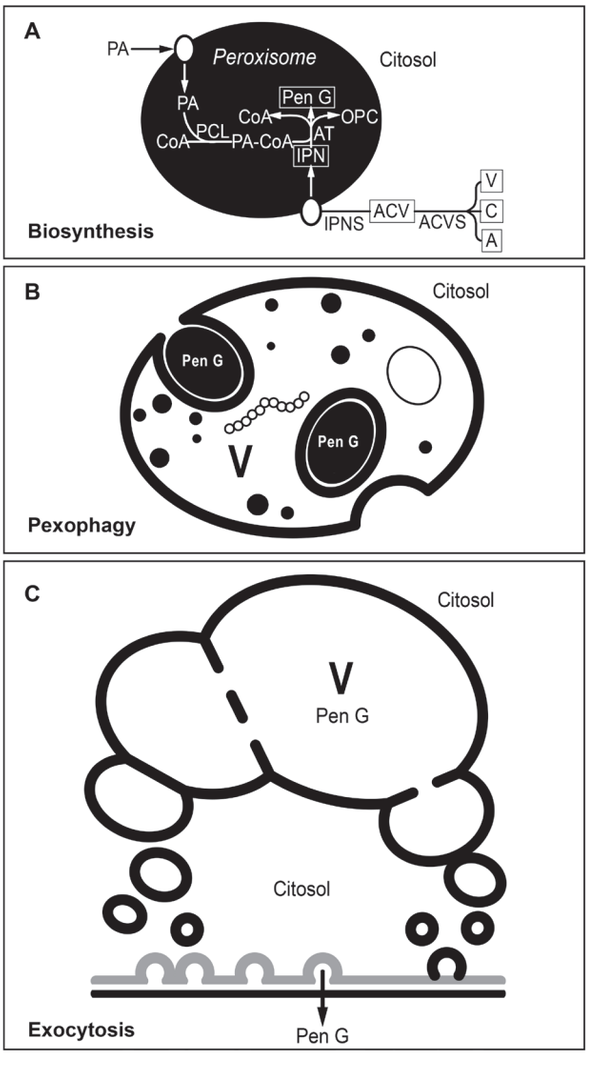

Fig. 2. A hypothetical overview of penicillin G secretion from the productive cells of the industrial mycelium of *P. chrysogenum* is depictured. (**A**) The process of penicillin G biosynthesis. The precursor amino acids L-α-aminoadipic acid (A), L-cysteine (C), L-valine (V) and the tri-peptide δ-L-α-aminoadipyl-L-cysteinyl-D-valine (ACV) as well as the ACV synthetase (ACVS) and isopenicillin N (IPN) synthase (IPNS) are located in the citosol. The last two enzymes of penicillin G biosynthesis, i.e. phenylacetyl (PA)-CoA ligase (PCL) and acyl-CoA:6-aminopenicillanic acid acyltransferase (AT) are located in peroxisomes were isopenicillin N (IPN) is converted to penicillin G (Pen G). In this process the 6-oxopiperidine-2-carboxilic acid (OPC) is produced by the conversion of the substituted side chain. (**B**) In the abundant vacuolar pexophagy, penicillin G is transported to the interior of vacuoles. (**C**) Finally, penicillin G is secreted from the vacuoles into the fermentation medium in the process of exocytosis, i.e. abundant cellular vacuolization, vacuolar budding and fusion of vacuolar vesicles with the plasma membrane. In spite of numerous attempts, at present, alternative ABC transporters for large-scale secretion of penicillin G by the industrial mycelia of *P. chrysogenum* are not known.

of the highest activity of penicillin G secretion from the peroxisomes to the fermentation medium. At the time of penicillin secretion abundant fusion of vacuolar vesicles with the cytoplasm membrane was observed [6]. The vacuolar vesicles are characteristically packed with organelle debris. In the mature non-growing hyphal cells of the low-yielding strain *P. chrysogenum* Q-176 a lack of large peroxisomes could be observed [5].

We suggest that the abundant pexophagy of large peroxosomes as well as the vacuolar budding observed in the productive and late-apical cells of *P. chrysogenum* PQ-96 might be directly involved in large-scale secretion of penicillin G. In these cellular arrangements the penicillin G formed in peroxisomes might be transferred to vacuoles and late secreted out of the cells by an exocytosis process. A hypothetical overview of penicillin G secretion from the productive mycelial cells of *P. chrysogenum* PQ-96 is shown in Fig. 2. The vacuolar pH about 5–6 is suitable for the stability of penicillin G. Our discoveries are consistent with the reported positive correlation between the number of large peroxisomes and penicillin G secretion [3, 4, 10, 11], as well as between the extended vacuolization and antibiotic secretion [6]. At a late stage of the cultures, peroxisomes are known to be integrated into vacuoles by the pexophagy phenomenon [4, 14].

7. Strain improvement

Selected strains of *P. chrysogenum* have been used for more than 65 years for the industrial manufacture of penicillins. Classical strain improvement has yield industrial strains that produce high titers of penicillin G [19]. Large-scale penicillin G production is a result of industrial strain improvement, including numerous mutagenesis and selection [17]. These strains contain up to eight copies of the penicillin G biosynthetic gene clusters (*pcbAB* gene, *pcbC* gene and *penDE* gene). The phenylacetyl-CoA ligase encoded by *phl* gene is not a part of the penicillin G biosynthetic gene cluster [19].

8. The future of β-lactams

Penicillin G is the first compound that was used in medical treatment beginning the era of antibiotic therapy. β-Lactams have been used extensively for treatment of various bacterial infections for more than half a century. Over many decades, the always increasing resistance of bacteria to β-lactams was forcing as to modify the chemical structures of these antibiotics to obtain novel compounds which are not accepted by β-lactamases and widely accepted by DD-peptidases. Inhibition of DD-peptidases is the mode of action of these antibiotics [5]. So far, we always modify the β-lactams to get efficient antimicrobial compounds, but as a response to our modifications the bacteria are doing exactly the same, modifying their mechanisms of resistance. Nevertheless, the search for novel β-lactams is at present the order of the day and should be mainly directed against the resistant DD-peptidases (not accepting the β-lactams) and the sensitive β-lactamases (accepting the β-lactams). At present, despite the widespread use over about sixty years the β-lactams are compounds with annual production volumes exciting 65,000 tons. The future of β-lactams must be protected by the correct medical application and novel strategies of treatment, e.g. the rotational use of β-lactams.

9. Conclusions

Our findings suggest that in penicillin G biosynthesis the structurally grouped organelles build the well-organized "machinery" composed of cytosol concentrated and membrane encompassed enzymes, substrates, intermediates, precursors, side- and end-products [7]. We have come also to the conclusions that in large-scale secretion of penicillin G the pexophagy phenomenon and the exocytosis should be currently considered as putative alternative for active secretion by the ABC transporters. Our ultra-structural findings [5, 6] provide evidence to support that pexophagy and exocytosis, i.e., vacuolar budding and fusion of vacuolar vesicles with the plasma membrane might be a major "machinery" of penicillin G secretion from the high-penicillin-producing cells of the industrial mycelium. We have also come to the conclusion that the sub-apical non-growing vacuolated cells are privileged in the biosynthesis and intensive secretion of Penicillin G. Moreover, the senescent mycelial cells are mainly involved in penicillin G secretion by the exocytosis process.

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