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MICROALGAE AS SOURCE FOR POTENTIAL ANTI-ALZHEIMER´S DISEASE DIRECTED COMPOUNDS - SCREENING FOR GLUTAMINYL CYCLASE (QC) INHIBITING METABOLITES

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ABSTRACT

Alzheimer´s disease (AD) is the most common form of dementia affecting predominantly elderly people from developed countries. One aspect of the illness is that patients suffer from an impaired memory due to deposition of aggregated Aβ-peptides forming amyloid plaques. According to the glutaminyl cyclase (QC) hypothesis this enzyme plays a key role in generating neurotoxic amyloid peptides (amyloid-β or Aβ) by modifying the N-terminus of peptides to N-pyroglutamated derivatives. These modified proteins are resistant to degradation and are at the same time "seeds" for the formation of toxic Aβ-oligomers in the brain. In order to screen for natural inhibitors of QC, strains of different species of algae belonging to Chlorophyceae and Eustigmatophyceae were cultivated in 100 L tubular photobioreactors. The resulting crude extracts of algae from exponential and stationary growth phases were tested for their inhibition properties of glutaminyl cyclase (QC). Here 27 of the 72 tested extracts inhibited the QC. Fractions separated by Sephadex G-15 column chromatography also showed QC inhibition activity.

KEY WORDS

Alzheimer´s Disease; Chlorophyceae; Eustigmatophyceae; Glutaminyl Cyclase (QC) Inhibitor; Screening.

INTRODUCTION

Alzheimer´s disease (AD) is the most common form of dementia characterized by neurodegeneration and neuroinflammation. The Alzheimer's disease International estimated that there are 46.8 million people living with dementia worldwide in 2014 (70% with AD), increasing to 74.7 million by 2030 and 131.5 million by 2050. The reasons are, firstly, the population growth and other demographic change [1].

Thus, there is a pressing need for new and efficient treatment strategies and more effective drug candidates to treat or prevent Alzheimer's disease. New causal approaches should be provided which halt or delay the progression of the disease which is not possible with today's available medications. Therefore it is important to understand the underlying molecular basis of Alzheimer's disease to find ways for causal treatment strategies.

Alzheimer's disease is characterized by neuron loss and the degeneration of synapses as well as neuroinflammation. In the brains of Alzheimer's patients, especially in the regions of the hippocampus and the neocortex protein deposits, the "amyloid plaques" and "neurofibrillary tangles" are formed. Because these areas of the brain are responsible for the higher mental abilities such as language and memory, a progressive loss of brain substance leads to increasing memory loss, confusion, and disorientation. Two other characteristics of this disease are constituted by a reduced acetylcholine level and an increased level of glutamate. These are the targets for previously approved drugs (inhibitors of acetylcholine and butyrylcholine esterase = AChE-I; NMDA-receptor antagonists = NMDA-RA) used in treatment of Alzheimer's disease. AChE-I such as donepezil (Aricept®), galantamine (Razadyne®), rivastigmine (Exelon®) and previously tacrine (Cognex®) typically delay worsening of symptoms for

an average of 6–12 months for about 50% of patients, and the NMDA-RA memantine (Namenda®) modulates glutamate activity and temporarily delays worsening of symptoms for some patients [2]. They mainly do not influence the formation of "amyloid plaques" and "neurofibrillary tangles".

The formation of amyloid deposits is based on a false splitting of the transmembrane amyloid precursor protein (APP = amyloid precursor protein) by the enzymes β-and γ-secretase [3]. The Aβ peptides, the main constituent of amyloid plaques, and various other metabolites are derived from APP by this false proteolytic cleavage [4, 5]. The further processed Aβ peptides possess at their N-terminus a glutamate residues, which can be modified to a pyroglutamyl residue (pE) leading to the Aβ peptides Aβ3 (pE)-40/42 and Aβ11 (pE)-40/42. This posttranslational pE-modification causes proteolytic resistance. The formation of the pyroglutamate-ring also increases the hydrophobicity at the N-terminus which in turn induces accelerated aggregation of the pEAβ-peptides. Several studies have shown that the senile plaques possess a prominent proportion of these neurotoxic pGlu-Aβ peptides [6]. Pyroglutamate-modified amyloid-β (Aβ3(pE)) peptides are gaining considerable attention as potential key participants in the pathology of Alzheimer disease (AD) due to their abundance in AD brain, high aggregation propensity, stability, and cellular toxicity [7]. N-terminally truncated and modified peptides are likely to be important for initiation of pathological cascades leading to AD. The formation of pGlu-peptides is catalyzed by glutaminyl cyclase, thus making QC to a valuable drug target [8].

First potential QC-inhibitors were identified and synthesized by Buchholz and coworkers. The structures are developed by applying a ligand-based optimization approach starting from imidazole [9]. Further classes of QC inhibitors were identified by homology modeling and afforded a first insight into the probable binding mode of the inhibitors in the QC active site. The efficacy assessment of the QC inhibitors was performed in cell culture, directly monitoring the inhibition of Aβ3 (pE)-40/42 formation [10]. Oral application of QC-inhibitors in two different transgenic AD mouse models and a Drosophila model reduced the Aβ3 (pE)-42 levels. Furthermore the treatment of mice reduced the production of plaque, gliosis formation and resulted in an improved performance in memory training and spatial learning tests [6, 8, 11-12,].

Further identification of QC inhibitors from different biological sources can help to broaden the diversity of the active compound classes and to enlarge the chemical space for drug development. Thus, algae were investigated for potential QC-inhibiting secondary metabolites. It is known that these organisms produce a range of bioactive compounds, which are not commonly available from other plants or animals. This enormous potential of algae is proven previously by a multitude of identified secondary metabolites with, for example, cytostatic, antibacterial, antiviral, anti-inflammatory, or antifungal properties, many acting via the specific inhibition of enzymes [13-19]. Thus some algae and secondary metabolites thereof can be expected to be a potential source of new QC inhibiting compounds. Therefore, we analyzed algal strains and crude extracts of different algae species belonging to Chlorophyceae and Eustigmatophyceae.

MATERIALS AND METHODS

Cultivation

Selected species of microalgae were cultivated fed-batch wise in a 100 L tubular photobioreactor 100 GS/PL at pH 7 using Setlik media (Setlik, 1967). The cultures were illuminated continuously with warm-white light (90 µmol/m2×s < OD 20; 150 µmol/m2×s >OD 20) at 28°C. Algae species belonging to Chlorophyceae (species 1-5) and Eustigmatophyceae (species 6) were obtained from SAG (Sammlung von Algenkulturen Göttingen, Germany).

A disc separator (Westfalia) was used for harvesting the biomass from the exponential growth phase (GP) and stationary growth phase (SP) of each species.

Extraction

The freeze-dried algal biomass of the exponential growth phase (GP) and stationary growth phase (SP) of each species were extracted by two solid-liquid-extraction procedures (Single solvent extraction and three step solvent extraction) using n-hexane, methanol and water.

In the single step extraction procedure in each case 10 g of all biomasses were grinded using sea sand and extracted triply repeated with 600 mL of *n*-hexane, methanol or water. The resulting extracts were labeled as *n*-hexane extract species 1 ***s***SP/GP ….6 ***s***SP/GP, methanol extract species 1 **s**SP/GP* ….6 **s**SP/GP* and water extract species 1 **s**SP/GP ….6 **s**SP/GP (Fig. 1a).

The successive three step extraction with increasing polarity from the same biomass was carried out for

each biomass by the extraction (triply repeated) with 600 mL of *n*-hexane using sea sand for grinding in the first step. After centrifugation (5 min, 2000 rpm) the *n*-hexane unextractable solid biomass was dried and extracted (triply repeated) with 600 mL of methanol in a second step. In the third step the dried biomass was extracted 3 times with water. The resulting extracts were labeled *n*-hexane extract species 1 ***m***SP/GP ….6 ***m***SP/GP, methanol extract species 1 ***m***SP/GP* ….6 ***m***SP/GP* and water extract species 1 ***m***SP/GP ….6 ***m***SP/GP (Fig. 1b).

The resulting *n*-hexane and methanol extracts were concentrated to dryness in a rotary evaporator under reduced pressure. For water extracts, an Infrared Vortex Evaporator (Zinsser Analytic, Germany) was employed.

The two extraction procedures resulted in a total number of 72 crude extracts.

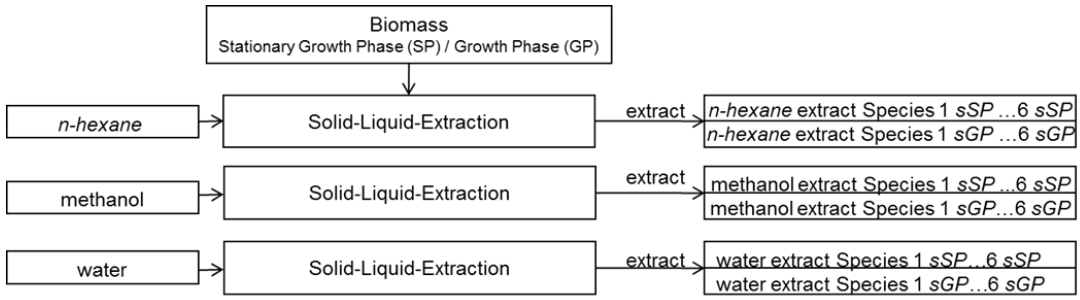

Fig. 1a Single solvent extraction
***methanol extracts: subsequence removal of chlorophyll by Chromabond SA-cartridges**

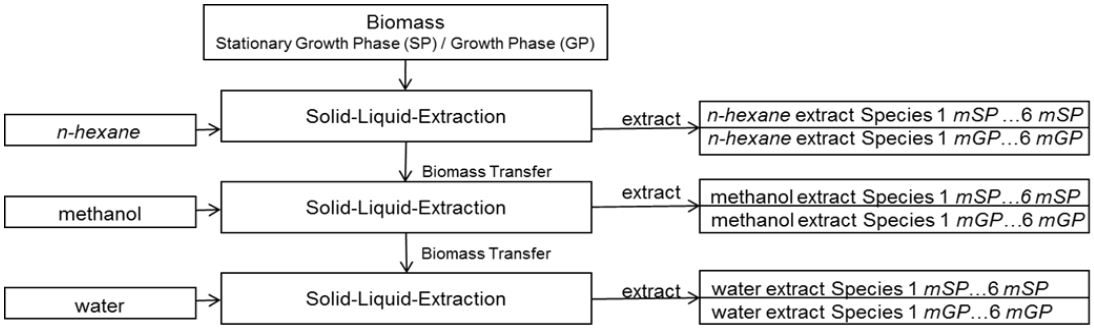

Fig. 1b Successive three step extraction with increasing polarity from the same biomass
***subsequence removal of chlorophyll by Chromabond SA-cartridges**

Sample preparation

The final crude *n*-hexane extracts were dissolved in DMSO for testing in QC-assay. Aqueous crude extracts were dissolved in water and insoluble particles were removed by centrifugation for assaying.

Due to the high chlorophyll content of the methanol crude extracts (methanol extract species 1 **s**SP/GP* ….6 **s**SP/GP* and methanol extract species 1 ***m***SP/GP* ….6 ***m***SP/GP*), Chromabond SA – cartridges (Macherey & Nagel) were used to remove this undesired metabolite(s) according to the company method (Macherey & Nagel Application database; http://www.mn-net.com). The resulting pre-cleaned extracts were evaporated to dryness and redissolved in pure methanol followed by a solid phase extraction on Chromabond C18eC – cartridge (Macherey & Nagel) using methanol as solvent. The obtained extracts were evaporated to dryness and dissolved in methanol for assaying. All extracts were prepared with a concentration of 1 mg×mL^{-1} for assaying.

Sephadex G-15 column chromatography

Positive assayed extracts were separated into 96 fractions (each fraction from 8 mL eluent) by column chromatography on Sephadex G-15 (Pharmacia Fine Chemicals Inc., NJ) column (3.2 × 62 cm) using 70 % methanol as solvent.

Glutaminyl cyclase (QC) inhibition assay

The determination of the catalytic QC-enzyme activity was accomplished by utilization of the fluorogenic substrate Gln-AMC (*N*-glutaminyl-7-amino-4-methyl-coumarin) and the supporting enzyme pyroglutaminyl aminopeptidase (pGAP) as well as purified human QC. For short, QC cyclizes Gln-AMC to pGlu-AMC which serves in a second step as substrate for pGAP. The

removal of AMC from pGlu-AMC is than detected at λ_{ex} = 380 nm; λ_{em} = 460 nm (Fig. 2) [20].

In this coupled optical assay the increase of the free AMC was continuously analyzed for more than 12 min at 30°C. The assay was conducted in microtiter plates by adding 100 µL 0.25 mM substrate, 50 µL 0.2 mg×mL^{1} extract, 25 µL supporting enzyme pGAP and 25 µL QC with a final volume of 250 µL per well. Because of the QC pH value dependency, 50 µL Tris buffer (0.1 M; pH 8) was added. The exact procedure was published by [21, 22].

All extracts and fractions were assayed in triplicate and classified as QC-active if inhibition activities were more than 20 % vs. control.

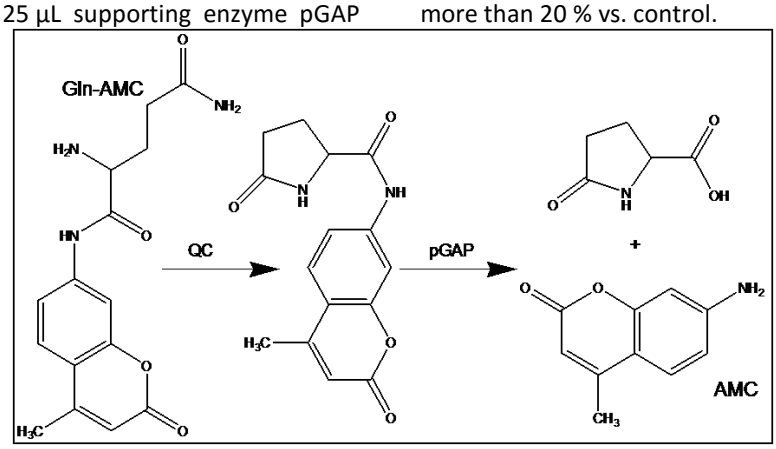

Fig. 2 reaction sequence of the QC assay

RESULTS AND DISCUSSION

Algae are chlorophyll rich organisms. For the successful bioassaying the removal of chlorophyll and its degradation products usually is a prerequisite, because the absorption of the dark green methanol crude extracts is interfering in photometric assays like the QC-enzyme assay. Thus a cartridge method to eliminate the chlorophyll was used. This method is fast, reproducible and offers high yields of the desired secondary metabolites. In comparison to a chlorophyll precipitation with citric acid buffer [23, 24], this more advanced method increases the yield of secondary metabolites up to 25-fold. However, certain organic cationic species may be retained too.

Altogether 72 crude extracts of 6 microalgae species in either their exponential growth phase GP or stationary growth phase SP were tested for their ability to inhibit human glutaminyl cyclase (QC). The results are given in Fig. 3 (only extracts with a relative inhibition of > 20 % are considered active).

Altogether 27 (38 %) of the tested algae extracts with final extract concentrations of 0.2 mg×mL^{-1} inhibit the glutaminyl-cyclase (QC). Among these, 5 *n*-hexane extracts show moderate inhibition of QC with activities between 24 % and 37 %. High levels of inhibition (48 % - 64 %) were obtained with three water extracts. In general, the chlorophyll-free methanol crude extracts constitute the most frequent and prominent inhibiton properties: 79 % of tested methanol extracts exhibit an activity between 21 % (moderate activity) and 71 % (high activity).

In summary, extracts resulting from algae`s growth phase (GP) are slightly more potent than extracts taken from the stationary growth phase (SP). Out of 27 positively tested extracts, 63 % (17 extracts) result from the biomass of the growth phase. Additionally, extracts from the single step solid-liquid-extraction procedure (**sSE**) proved more potent as extracts from the three-step extraction **(mSE).**

In order to exclude unspecific enzyme inhibition, the 24 methanol extracts were also tested at a higher final extract concentration of 2 mg×mL^{-1} (Fig. 4) which causes QC inhibition up to 99 % (Species 1 sGP, Species 2 sGP, Species 2 sSP, Species 4 sGP, Species 4 sSP, Species 5 sGP), i. e. the inhibition basically is concentration-dependent as required by theory.

To identify QC-inhibition metabolites, the active methanol extracts of species no. 3 were further separated by Sephadex G-15 column chromatography into 96 fractions as described above. This crude separation let to a moderat concentration of activity peaking in fraction 72 with an inhibition activity of 66 % (Fig. 5), vs. an activity of the total extract of 57 %. However, no distinct separation of active constituents is possible by this chromatographic technique alone. In order to identify single active compounds, more advanced separation techniques will have to be applied in the future.

Glutaminyl cyclase (QC) is an emerging new and promising target for the causal treatment of AD. Accordingly, there is an increasing interest in exploring new and potent QC-inhibitors, especially if they are of natural origin. Until now, algae as a potential source of QC-inhibiting compounds have not been investigated. Only compounds acting as inhibitors of acetylcholine esterase (AChE) - a known target to treat Alzheimer's disease - have been isolated [25]. A particularly active substance class of these are Phlorotannins from macroalgae. The isolated compounds Eckol, Dieckol, 2- Phloroeckol and 7-Phloroeckol from *E. stolonifera* show a concentration-dependent inhibition of AChE [26, 27]. Phlorotannins isolated from *Eisenia bicyclis* inhibit the BACE-1 (APP-cleavinge enzyme), which plays an important role in the APP processing [28]. Also a BACE-1 inhibiting linear depsipeptide (Tasiamide B) was isolated from cyanobacteria [14].

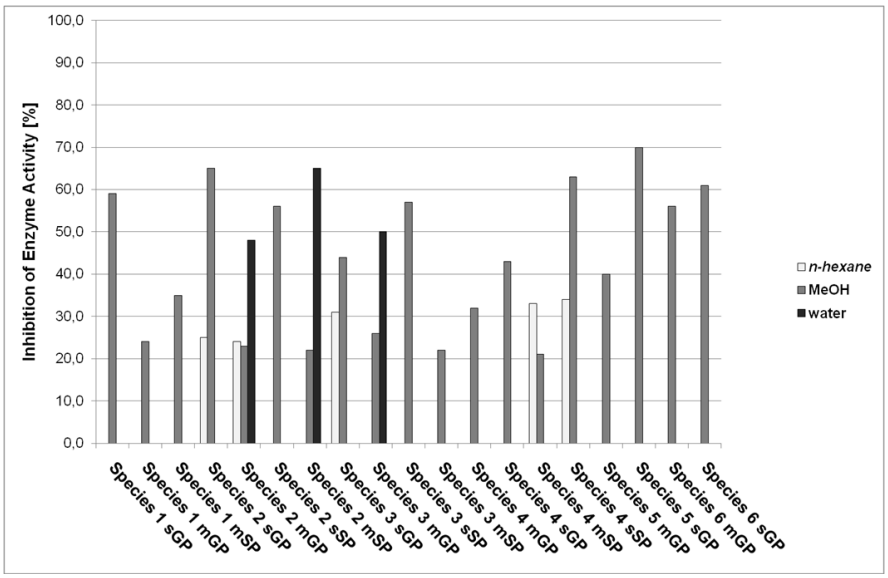

Fig. 3 Inhibition activity of crude extracts tested positive against QC: 27 of 72 crude extracts from 6 different species tested with a final extract concentration of 0.2 mg×ml^{-1}.

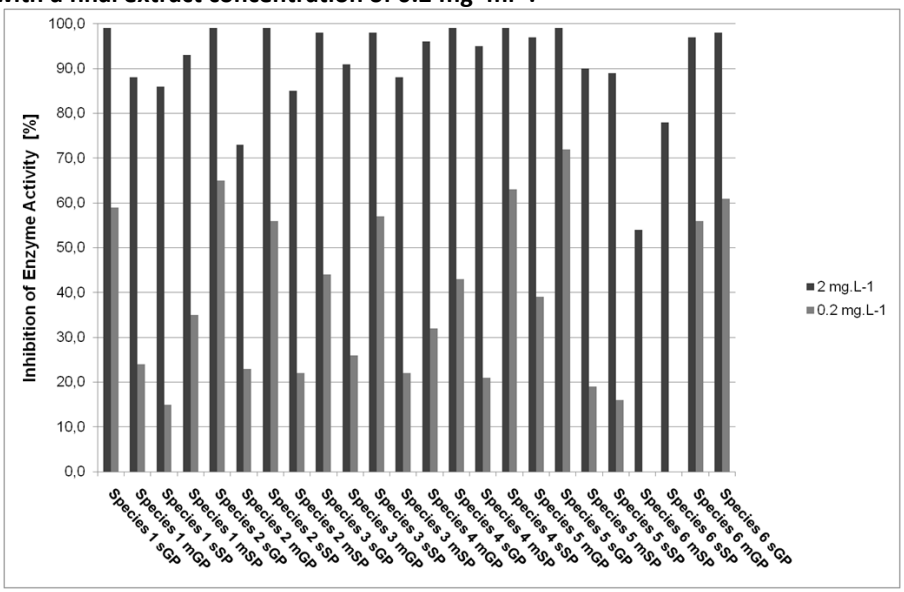

Fig. 4 Simple concentration dependency of the inhibitory activity of methanol crude extracts against QC.

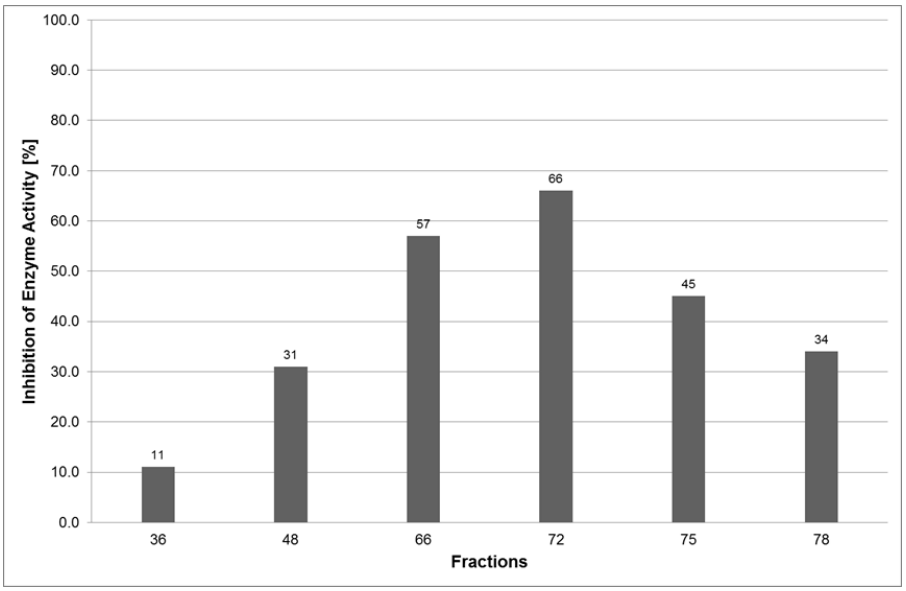

Fig. 5 Partial concentration of the active principle(s) in the middle fraction on sephadex G15.

CONCLUSION

In toto, 1/3 (27 extracts) of extracts from algal species belonging to Chlorophyceae (species 1 - 5) and Eustigmatophyceae (species 6) contain bioactive material which can be used as a starting point for potential compound development. The screening results demonstrate that algae are also able to produce secondary metabolites with inhibiting activity against glutaminyl cyclase. Methanol extracts of several algae strains seem to be particularly rich in effective compounds. They inhibit the QC-enzyme concentration dependent and will serve as basis for further bio-guided isolation efforts to identify the active principles.

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