Exploring the properties of a newly described rotifer-specific biopolymer escpecially in relation to neurotoxic aggregates

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PhD Thesis

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Publications

I. **Balazs E,** Galik-Olah Z, Galik B, Bozso Z, Kalman J, Datki Z, Neurodegeneration-related beta-amyloid as autocatabolism-attenuator in a micro-in vivo system, IBRO Reports, 2020. https://doi.org/10.1016/j.ibror.2020.10.002, MTMT: 31639846, **IF: 0.5**

II. Datki Z, Acs E, **Balazs E**, Sovany T, Csoka I, Zsuga K, Kalman J, Galik-Olah Z. Exogenic production of bioactive filamentous biopolymer by monogonant rotifers, Ecotoxicol Environ Saf 2021. https://doi.org/10.1016/j.ecoenv.2020.111666, MTMT: 31776926, **IF: 7.129**

III. **Balazs E,** Galik-Olah Z, Galik B, Somogyvari F, Kalman J, Datki Z, External modulation of Rotimer exudate secretion in monogonant rotifers, Ecotoxicol Environ Saf 2021. https://doi.org/10.1016/j.ecoenv.2021.112399, MTMT: 32059771, **IF: 7.129**

IV. Datki Zs, **Balazs E**, Galik B, Sinka R, Zeitler L, Bozso Zs, Kalman J, Hortobagyi T, Galik-Olah Z, The interacting rotifer-biopolymers are anti- and disaggregating agents for human-type beta-amyloid *in vitro*, International Journal of Biological Macromolecules, 2022. https://doi.org/10.1016/j.ijbiomac.2021.12.184, MTMT: 32591465, **IF: 8.025**

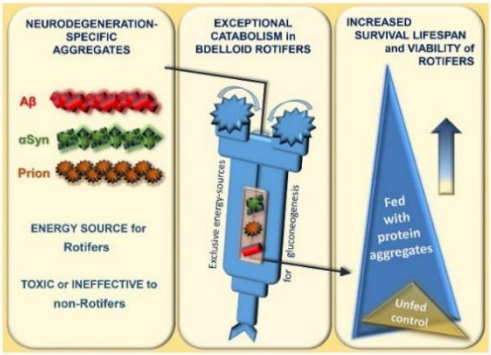
Abbreviation

Aβ → beta-amyloid; **Aβ42** → beta-amyloid 1-42; **BisANS** → 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid dipotassium salt; **BSA** → bovine serum albumin; **ConA** → Concanamycin A; **D0** → Day 0; **D20** → Day 20; **D25** → Day 25; **DW** → distilled water; **ED** → *Euchlanis dilatata*; **ED-RIC** → *Euchlanis dilatata* Rotimer-Inductor Conglomerate; **EDTA** → ethylenediaminetetraacetic acid; **FROS** → functionally reversible organ shrinkage; **FROSi** → FROS index; **FTIR** → Fourier Transform Infrared Spectroscopy; **H-Aβ** → human-beta-amyloid; **LB** → *Lecane bulla*; **LB-RIC** → *Lecane bulla* Rotimer-Inductor Conglomerate; **NaOH** → sodium hydroxide; **PI** → propidium iodide**; RIC** → Rotimer-Inductor Conglomerate; **RPC** → RIC-producing capacity; **S.E.M**. → standard error of the mean; **S-Aβ42** → scrambled isoform of Aβ; **SEM** → scanning electron microscope; **ThT** → Thioflavin T.

1. Introduction

Neurodegenerative disorders, such as Alzheimer's disease (AD), could be regarded as phenotypes secondary to the progressive functional impairment of proteomes. The molecular basis of brain aging can be described as an accelerated accumulation accompanied by reduced clearance and degradation of misfolded proteins. There is a direct correlation between protein aggregation and age-related pathologies. Intramolecular domains arranged in a β-sheet conformation are highly resistant to enzymatic action. The various neurotoxic aggregates, share common features, with their accumulation and aggregation facilitating neurodegeneration, where the aggregation is caused by an abnormal conformational change in related molecules. These dysfunctions occur either extra- or intracellularly. Misfolded peptide and protein aggregates can be partially digested by several endogenous enzymes, beta-amyloids, such as Aβs, are central molecules in aging-associated diseases, representing an initial point in the development of dementias. The rotifer is one of the most commonly used microinvertebrate animal models; these animals are excellent models of ecotoxicology, aging, pharmaceutical, or longevity-related research. The rotifers are validated models of toxicity screening related to environmental parameters and chemical agents. Although their bodies consist of approximately 950-1000 cells, they have complex organ systems, including gastro-intestinal tract, reproductive organs, nervous system, or secretory glands. They are promising scientific models due to their optimal culturing requirements, short lifespan and specific measurable phenotypic features and viability markers. A relevant example of this is the phenomenon that some rotifers can exceedingly metabolize (catabolize) the extremly resistant peptide- or protein aggregates (well-known neurotoxins), such as alpha-synuclein, scrapie prions and beta-amyloid (Aβ) under starvation conditions. In an exceptional way, the human-type aggregates are potential nutrient sources to bdelloid rotifers. These animals can use conglomerates and aggregates as an energy source, by their phylogenetically selected ability (**Fig. 1**).

Figure 1. Exceptional in vivo catabolism of neurodegeneration-related aggregate by rotifers



Bdelloids, have high tolerance to normal environmental changes due to their capability of adaptive phenotypic plasticity. One of the definitive proofs of the adaptability of these microinvertebrates is their ability to secrete biopolymers. Biopolymers are produced exclusively by living organisms, and these chemical substances are composed of repetitive units that create higher-order molecular structures. They can be grouped based on their chemical structure. The structural formation of polymers always requires cross-linkers, such as metal ions, e.g., calcium. The variety of biopolymers is shown by the fact that different animals are able to secrete versions with other functions depending on their lifestyle and environment. Due to the favorable properties of these natural materials, such as antimicrobial or hypoglycemic activities, they are used in multiple fields for pharmaceutical, medical, or industrial purposes. As the eco-friendly approach becomes dominant in industrial developments, the relevance of biopolymers gains more importance due to their biodegradable, renewable and environmentally friendly nature. These natural agents are applied in environmental protection, agriculture, food-, pharmaco- and energetic industries. Because biopolymers are biocompatible, biodegradable and have low immunogenicity, they are promising theoretical sources in biomedicine, even in neurobiology. There are several natural molecules (e.g., collagen, gelatin, heparin or chitosan) that are the bases of artificial products available in clinical application. Furthermore, many plant-based biopolymers (e.g., cellulose) can also be used as medical engineering constructs due to their excellent physical and biochemical properties. These agents are suitable as therapeutic molecules in drug and gene delivery.

Many studies deal with biopolymers, but we found no relevant data in the literature about the secretion of monogonant or bdelloid rotifer biopolymers. At the same time, they are characterized by outstanding biological diversity, with many new properties and phenomena waiting to be explored. The goal of our research team is to learn as much as possible about the natural characteristics of these animals.

2. Specific aims

The physiological development of neurodegenerative diseases is the consequence of the accumulation of toxic aggregates in the brain. During our previous research, our group discovered a unique phenomenon, according to rotifers, as micrometazoans, which are able to metabolize various neurotoxic aggregates, including human-specific beta-amyloid, and consume them as food. Our main goal was an explorative investigation of the background of this ability. Specific aims:

1. Further investigation of the exceptional catabolic ability of rotifers, understanding of the interaction between animals and neurotoxic beta-amyloid.
2. The theoretical interpretation of the background mechanism of the above-mentioned capability, the assumption of the existence of a special molecule that helps the degrading enzymes and modifies the aggregated structures.
3. Searching for biomolecules secreted by rotifers (consisting of approximately 1000 cells but with complex organs), finding presumed exudates.
4. Development, testing, validation, and application of methodologies and tools necessary to implement pioneering interdisciplinary experiments.
5. Explorative examination of the potentially founded biomolecules, produced in relatively large quantities, both alone (e.g., bioactivity, environmental regulation) and together with targeted human-type aggregates (molecular interactions).
6. Setting up a complex theory to explain the unique ability of rotifers regarding the catabolism of toxic aggregates.

3. Material and methods

3.1. Rotifers

3.1.1. Origin of animals

During measurements were carried out on invertebrate rotifers, *Philodina acuticornis, Adineta vaga*, *Euchlanis dilatata* and *Lecane bulla* species; therefore, no specific ethical permission was needed according to the current international regulations. The measurements were carried out in accordance with globally accepted norms: Animals (Scientific Procedures) Act, 1986, associated guidelines, EU Directive 2010/63/EU for animal experiments, and the National Institutes of Health instructions for the care and use of Laboratory animals (NIH Publ. No. 8023 from 1978).

3.1.2. Identification of pieces

The further species were *Lepadella patella, O. F. Muller, 1773; Colurella adriatica, Ehrenberg, 1831; Itura aurita, Ehrenberg, 1830; Trichocerca iernis, Gosse, 1887; Brachionus leydigii rotundus, Rousselet, 1907; Brachionus calyciflorus, Pallas, 1766; Cephalodella intuta, Myers, 1924; Synchaeta pectinata, Ehrenberg, 1832*.

3.1.3. Animal culturing

All tested rotifers are cultured in flasks at 23°C, under diffused light, in the standard medium nutrient solution.

3.1.4. Harvesting of animals

The first step in isolating *E. dilatata* and *L. bulla* is to filter rotifers from flasks (more vigorous shaking). Filtration is performed with a double filter (1st filter: 500 μm Ø; 2nd filter: 80 μm Ø (pore diameter). The process of isolating *P. acuticornis* and *A. vaga* is first picking up the substrate with a pipette, then transferring the bdelloids to a well plate.

3.1.5. Metabolism-related *in vivo* experiments

The metabolism-related measurements in monitoring the size of the germovitellaria in the presence of glucose started on Day 0 (D0), providing a reference control. After twenty days (day 20; D20) of starvation and five days (day 25; D25), organ regeneration was shown. On D20, one-time feeding (600 μg/mL yeast homogenate) was applied and followed-by five days of recovery. The shrinking process with functional (egg production) regeneration capacity was named "functionally reversible organ shrinkage" (FROS). To investigate (n = 5, well) the amount of protein in the animals, BisANS was applied parallelly with detecting the total amount of nucleic acid, where PI was used after 10 min incubation and washing.

3.2. Rotimers

3.2.1. Biopolymers and their conglomerates

3.2.1.1. Induction

Secretion occurs only when the cilia of the rotifer are mechanically irritated with inert particles of various types and sizes. Inductors can be **a.** yeast: heat inactivated cell skeleton; **b.** BSA: heat-denatured (den-BSA); **c.** epoxy: metal beads coated with polymers; **d.** Carmine: mechanically powdered crystals (with electric coffee grinder); **e.** urea: ultrapowdered crystals; **f.** cellulose: powdered micro-cellulose. Secretion of particle-induced biopolymer resulted a ‘Rotimer-Inductor Conglomerate’ (RIC) in a high-density web form after 20 min incubation time (**Fig. 2K-M**; with yeast inductor). After carefully removing the well solution, these RIC products were desiccated (dried) at room temperature (25 °C) and at 40% humidity in the dark for 30 min. The RIC analysis was used to select the best RIC-producing species using the yeast as an inductor related to RIC-producing capacity (RPC); furthermore, the RPC was also applied for screening different Rotimer inductors and rotifer-influencing factors (with epoxy inductor). The entities kept in Petri dishes were treated in populations during RIC production experiments, then they were rinsed twice by standard medium. The treated and tested animals were selected, and their RIC production was monitored. During repeated inductions, there were 10-minute breaks between treatment rounds. After each round, the same rotifers were carefully transferred with a micropipette to a new well to initiate the next cycle.

3.2.1.2. Regulation

We examined the production of Rotimer under various environmental factors, such as temperature, pH, metal ions, and different pollutants, the two species examined during the experiment were *E. dilatata* and *L. bulla*. Every measurement was performed in standard environment (24 °C, pH = 7.5, 40% air humidity, in standard media and 12:12 hours dark-light), except for the actual parameter of interest or the optimized experiments (for *E. dilatata*: 22 °C, pH = 7.8; for *L. bulla* 25 °C, pH = 7.2).

3.2.2. Isolation of conglomerates

In the case of the Carmine inductor used during Rotimer production, we first draw off the medium with the help of a 5 ml pipette, which is attached to the base of the petri dish wall at an angle of 45 °C. The RIC attached to the bottom of the petri dish is then picked up and homogenized with 4 ml of DW. Then the rotifers remaining in the solution are removed by filtration using a funnel. Next, filtration is carried out directly into a 2 ml eppendorf, followed by centrifugation (35000 g/12 min; 4 °C), carefully removing the supernatant and maximizing the pellet volume in 150 µl DW. When using epoxy beads, the first steps until the removal of rotifers are the same as when using the Carmine inductor mentioned above. A solution free of animals but containing RIC was then prepared at room temperature, and the exudate-coated beads were reversibly fixed using a DynaMag-2 magnet.

3.2.3. Storage of conglomerates

The Rotimer-Inductor Conglomerate is stored in a 500 µL eppendorf supplemented with 150 µL DW at -70 °C.

3.2.4. Biochemical investigation of Rotimers

3.2.4.1. Analysis of Rotimer with FTIR spectroscopy

The FTIR spectroscopy method was used for the biochemical analysis of Rotimer in order to perform measurements, Rotimer containing RIC samples were prepared.

3.2.4.2. The Rotimer detection with scanning electron microscope (SEM)

Rotimer formations (glues and fibers) appeared undetectable under inverted light microscopy; therefore, analysis was performed with a scanning electron microscope (**Fig. 2**; SEM). For this series of experiments, the best and smallest stimulator, epoxy, was used (50 µg per mL) to induce Rotimer secretion and RIC formation in the most prolific species, *E. dilatata*. The samples were coated with ionic gold. The preferred areas (based on their quality) were subjected to SEM. The sample-carrier coverslip was fixed onto a stub using carbon tape. The coverslips were coated with gold using a Quorum Q150R sputter coater for two min.

3.2.4.3. Chemical influence on the integrity of RIC web

During our work, we examined the influencing role of various chemical substances on the dissolution of Rotimer *in vitro*. Before investigating the inductor-cohesive-stability of Rotimer to reveal the structure of RIC (Carmine inductor, except of NaOH, where it was Epoxy), the animals were carefully

removed by a micropipette. Then, the well-content was supplemented by the 10x concentration of treatment agents (enzymes, solvents, chelators, alcohols, and pH-solutions).

3.2.5. Biological investigation of Rotimers activity

3.3.5.1. *In vivo* bioactivity

Viability tests related to the bioactivity of Rotimer were first performed *in vivo*. In all viability-related experiments, based on Calcein-AM (5 μM), the labeling interval was 1h at room temperature in the dark. The viability (cell-fluorescence) and motility (movement of cells) of algae and yeast cells were measured in separate 24-well plates. Confluent cell population was applied in cytoplasmic calcium detection where the fluorescence intensity (FI) of intracellularly trapped Calcein was detected.

3.3.5.2. *In vitro* molecular interactions

A further biological activity-related study of the molecular interaction of RIC and H-Aβ was carried out under in vitro conditions. Based on the manufacturer's product description of the Dynabeads M-270, 1 mg of these beads can bind approximately 10 ± 2 μg of ligands (in this case the Rotimer); however, 6 mg of beads from one induction can theoretically bind about 50-60 μg Rotimer onto their surface if they are widely covered. The working concentration of Aβs was determined to be 50 μg/mL. During the investigation, we first used a stagogram-based optical method. Then, after a short time (5 min) of incubation, the interaction between mixed monogonant-specific RIC (6 mg Rotimer-coated epoxy beads per mL) and three hours (3h) or three days (3d) Aβ aggregates was investigated.

3.2.6. Rotimer-related depletion

The potential role of Rotimer against Aβ42 aggregates was investigated in four different species (*E. dilatata; L. bulla; C. intuta; S. pectinata).* Concentration of Aβ42 stock solution was 1 mg/mL (DW) with 3h aggregation period. Neutralization (to pH 7.5) of this solution was performed with NaOH (1 N). After 10-fold dilution with standard medium, the final (working) concentrations were 100 μg/mL. After harvesting, 30 ± 2 mature rotifers per well (n = 24 well/species in 96 well-plate) were treated in 0.2 mL volume. The treatment period was 5 days in standard conditions. The depletion protocol was performed before the Aβ42 was administered and this protocol was in line with the one applied in Rotimer production factors treatment.

3.3. Statistics

The error bars show the standard error of the mean (S.E.M.). Next, the one-way ANOVA was applied for statistical analysis, followed by the Bonferroni *post hoc test*. The homogeneity and normality of the data were checked, and they were found suitable for ANOVA followed by Bonferroni post hoc test. The different levels of significance are indicated as follows: $p^{*,\#} \leq 0.05$, $p^{**,\square\square} \leq 0.01$, $p^{***,\#\#\#,\square\square\square} \leq 0.001$ and $p^{****} \leq 0.0001$ (all marks are defined in the given figure legend).

4. Results

4.1. The Rotimer-Inductor Conglomerate

4.1.1. Production

The rotifer-specific biopolymer, namely Rotimer, was first described in the literature by our research group. This exudatum was observed in six (**Fig. 2A-F**) different monogonants: *E. dilatata, L. bulla, L. patella, I. aurita, C. adriatica* and *T. iernis*. Rotimer is a special biomolecule complex that is vital for the survival of the animals that produce it. After constant laboratory cultivation, *E. dilatata* and *L. bulla* proved to have the six investigated species' most intensive RPC. Representative photo (**Fig. 2K**) about RIC formation produced by *E. dilatata* was taken.

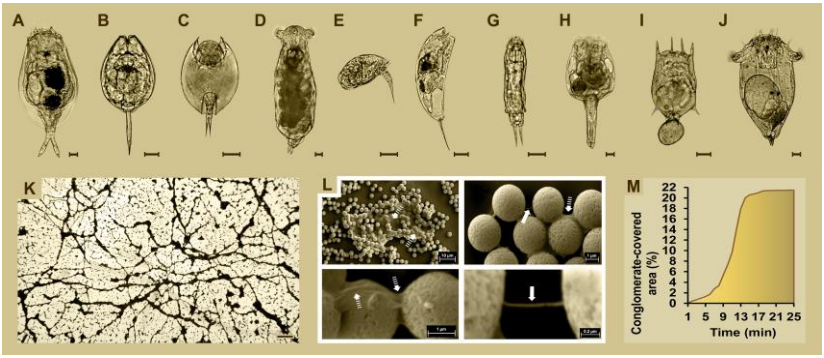


Figure 2. Presentation of the rotifers and the rotifer-specific biopolymer (Rotimer). *The representative photos of monogonant rotifer species (A-F) are shown. The species used in experiments related to Rotimer-related biomaterial (scale bar: 20 μm): E. dilatata, A; L. bulla, B; L. patella, C; I. aurita, D; C. adriatica, E; T. iernis, F; C. intuta, G; B. leydigii rotundus, H; B. calyciflorus, I; S. pectinata, J. The representative figure shows the network structure of the "Rotimer-Inductor Conglomerate" (RIC) formed by E. dilatata, K (scale bar: 200 μm). The SEM photos (L) show different occurrences of Rotimer after epoxy induction (scale bar: 0.2 and 1 μm). Regular arrows indicate the filamentous form, while dashed arrows indicate the glue-like structure. The kinetics of RIC production (M) was measured by the saturation (%) of the area covered by RIC as a function of time (minutes).*

The filaments drawn by *E. dilatata* monogonants are fragile (33 ± 3 nm in cross-section) and can only be observed only by SEM. Secretion of Rotimer could be mechanically induced by approximately 2.5 to 50 μm diameter particles, respectively in *E. dilatata*. No RIC production was observed above or below this range. Biopolymers are secreted very quickly (4 μm/sec) and bind to the surface of the inducers. The secreted exogenic Rotimer (**Fig. 2L**) can either be filamentous (arrow) or gluelike (dashed arrow).

Rotimer was secreted by six different rotifers and this product was investigated to assess which species could be the most effective producers under yeast induction.

4.1.2. Analysis

The Rotimer-Inductor Conglomerate web seemed to be a very complex formation and it is characteristic to some monogonants, we wanted to test how the different environmental factors influence its production. Since yeast is part of the rotifers' diet, it was also optimal as an inducer. After successive inductions, depletion of endogenous Rotimer sources was observed in *E. dilatata*. After the third round of stimulation, the size of the conglomerates did not change significantly, but the proportion of the area covered by the conglomerate decreased. Differences between both measured parameters were found in the first-second rounds and third-fourth rounds. Presumably, the animals ran out of resources for production and selection, and there was not enough time for production. Active synthesis of Rotimer is required for RIC formation, which is an energetically active process.

After starvation, we observed a second mode of depletion of Rotimer secretion due to the lack of nutrients. Changes in the monitored parameters were observed between the RIC production after the first-second day of starvation and the third-fourth day. Since food was unavailable for the animals, they presumably used the endogenous Rotimer substrate as an energy source. The regeneration time of RIC production capacity was 30 ± 4 min in *E. dilatata* entities exhausted from starvation. In the presence of cellulose, carmine, urea, and Epoxy beads, there was no RIC production in the depleted rotifers for longer than 6 hours. Rotimer production in rotifers is relatively fast and highly nutrient-dependent.

Surprisingly, the lower temperature (10 °C) did not decrease the monitored parameters compared to its controls, while the higher temperature (35 °C) was able to stimulate production. Similarly, to RIC production, higher temperatures favored monogonants. Immediately after the isolation of *E. dilatata* entities, increasing (1 g/L) or decreasing (DW) salt concentration had no immediate effect on RIC production compared to the standard medium; however, when these animals were left to rest (washed) in completely ion-free medium for half an hour, the RPC disappeared. In contrast to the comprehensive data on the salt sensitivity of monogonants, we found that extreme osmolarity has no adverse effect on the monitored parameters. During this study, one of the measured parameters was the average size of the conglomerate. Still, during another experiment, we extended the examination of the parameters with a higher resolution (yarn length), which is described in the regulation section. Upon further investigation, we saw how RIC could maintain its integrity in the presence of various chemical agents. We hypothesize that hydration and metal ions may be required to maintain Rotimer integrity. It is challenging to examine the components and structure of Rotimer, as we currently cannot separate the biopolymer from the specific inducer.

4.1.3. Regulation

Rotimer production is sensitive to environmental factors, such as temperature, pH, metal ions, and various pollutants. These influencing factors have correspondingly different effects on rotifers; however, each species has its optimum. To accurately assess the calcium dependence of RIC production,

dose-efficacy was measured. Sodium does not affect secretion production; the various doses of calcium were supplemented with it, the same as the amount of metal ions, like standard media. To avoid dose dependency, it is essential to ensure a constant osmolarity in the given artificial medium. The inhibitory effect of non-membrane permeable calcium-specific chelator (EDTA) on RIC production proves that this process happens extracellularly, presumably at the inlet of the intestinal tract. The calcium dependency of biopolymer-secretion and formation is not a rare phenomenon in the animal kingdom, especially in aquatic invertebrates.

If exudate secretion was induced under species-specific optimal conditions in a modified culture medium, the length of filaments and the RIC amount represents that *E. dilatata* can be better potentiated than *L. bulla*. It was shown by non-cell permeable Fluo-3 fluorescent dye that significantly more calcium was used by the animals during RIC production than without its induction. These data suggest that rotifers bind metal ions and extract them from their environment.

The two examined species *E. dilatata* and *L. bulla*, show different reactions to environmental factors (temperature, pH) compared to each other (**Fig. 3**), except for the presence of calcium and the modulating effect of various drugs (Lucidril).

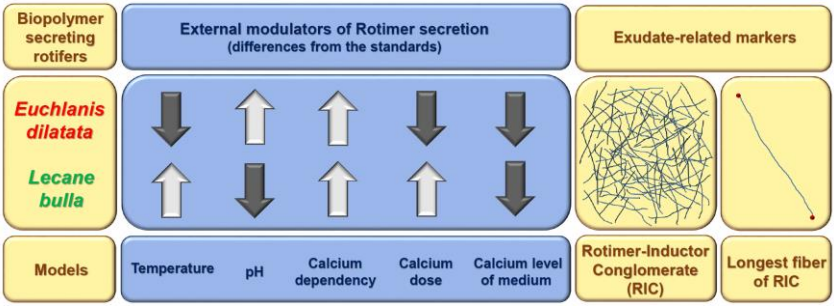


Figure 3. External modulators of Rotimer secretion summary table

4.1.4. Bioactivity

The physiological effects of Rotimer produced by *E. dilatata* were investigated on three different cell types. We used Den-BSA as an inducer, also found in the standard medium of classical cell culture; therefore, it had a no different physiological effect on the cells in the system. The average movement of the cells was well measurable in untreated controls. Passive (diffusion-based) and active (cell movement) motility occurred in yeasts and algae, while only dynamic localization was detectable in human neuroblastoma. RIC only slightly reduced the viability of the tested cells, where the extent of the damaging effect did not reach the LD50; on the contrary, it inhibited their motility in all cell types.

4.1.5. Conglomerate-aggregate interactions

During the interaction between the RIC and the aggregates, we investigated the binding between the biopolymer-containing conglomerates and H-Aβ neurotoxic aggregates, respectively, to reveal the effects of the RIC complex on H-Aβ aggregation itself. The connection between H-Aβ and *E. dilatata/L. bulla*-RIC (ED/LB-RIC) was investigated by stagogram analysis which is a simple, but widely accepted method to visualize e.g. the aggregation capacity of different molecular composition of samples, based on their optical diversity. In this case, the active ingredient was RIC (epoxy beads with Rotimer). The solutions of fresh (0 min) H-Aβ and S-Aβ (with random sequence of amino acids) were not presented on the stagogram figure, because they showed transparent crystallizing patterns; therefore, the border lines of the dessicated samples could not be identified.

The crystallized droplets of the groups differed in both density and structural phenotype. This is a closed and exactly defined chemical environment with few components; however, it is a well controllable *in vitro* system. The more aggregated (3d) H-Aβ showed a denser pattern compared to the few hours-incubated ones (3h). Both RIC groups of the two tested animal groups presented outstanding density in the empiric analysis of stagograms; moreover, they provided a maximal reference to the further analysis of the other samples. The density of ED-RIC-H-Aβ conglomerate is higher compared to LB-RIC-H-Aβ conglomerates. Based on the results, it can be declared that are possible molecular interactions between Rotimer and Aβs, particularly with H-Aβ form. The relations between RIC and H-Aβ of both rotifer species showed similar tendencies, however, we also noticed that the samples originated from *L. bulla* the pictures were significantly brighter.

In addition to the optical analysis, the fast interactions between the investigated molecules were also analyzed using fluorescence methods. For measuring the indicator fluorescent signals two adequate dyes were applied, the Bis-ANS and the ThT. The H-Aβ (3h) with lower organization contains oligomers in higher proportions and they were measured by Bis-ANS, while the considered fibrillar ones with longer incubation time (3d) were detected by ThT. In anti- and disaggregating measurements all samples were detected with both fluorophores. The results of the interaction studies can be classified into three units: 1. H-Aβ-types alone; 2. inductor, various RIC-versions and the inductor-Aβ interactions as relevant controls; 3. the interactions between the species-specific RIC and the different H-Aβs (specified in amino acid sequence and levels of aggregation). Based on the followed-up aggregates, there are three categories: 1. with Aβs alone; 2. Free Aβs in the supernatant; 3. binding Aβs to the RIC-types.

The H-Aβ interacted with the conglomerates of both rotifer species and relatively high fluorescent signal was shown, while the S-Aβ gave more lower value similarly to the background control. The data derived from this reference amyloid type, made us to conclude that there is sequence specificity in H-Aβ-binding to biopolymers. The H-Aβ samples with lower (3h) or higher (3d) conformational organization were significantly bound to tested Rotimer

coatings, but it showed higher affinity to the biopolymer secreted by *E. dilatata* than the one by *L. bulla*.

The materials from *E. dilatata* were more effective in anti-aggregation processes, than the ones derived from *L. bulla* using the same experimental time. The 3d group was the less efficient. In the LB-RIC and H-Aβ/3d combination the ThT gives an elevated level; thus, unlike the other samples, here was no clear conclusion about the aggregation level of H-Aβ.

Based on the characteristics of the two fluorophores, we may assume that the presence of Rotimer in the samples have disaggregation effect against the H-Aβ.

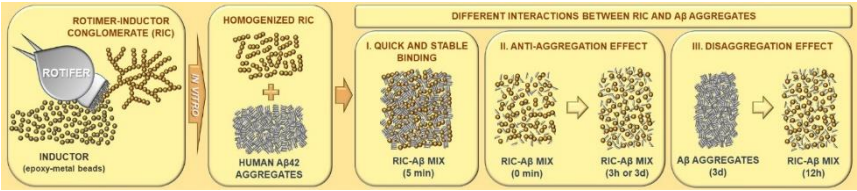


Figure 4. Interactions between rotifer-RIC and human-type Aβ aggregates

4.2. Rotifers and beta-amyloid relations

4.2.1. Autocatabolism

The Aβ42 is a well-known neurotoxin, which is prone to form highly resistant aggregates in an aquatic environment. The bdelloid rotifers can catabolize these aggregates, with no physiological damage. Our aims were to reveal the special role of Aβ42 in autocatabolism-related processes. To identify the sequence specificity of this pathological molecule, we used its coded version as a control. The molecular weight of S-Aβ42 was the same as that of the wild-type form, with a different sequence of amino acids.

To connect the autocatabolism-related processes with starvation-induced organ shrinkage, we applied ConA during the experiments. In conclusion, we can state that the total protein amount decreased on the 20th day, indicating that the animals catabolize them for survival. The amounts of nucleic acid did not show any changes in either species. In line with the adequate investigations, we detected significant increase in autocatabolism-related vesicular acidification in relevant species on D20 compared to the untreated reference values of D0 phenomena are appropriate empirical and physiological markers of autolytic metabolism in the rotifers.

In FROS-related measurements the ConA, Aβ42 or S-Aβ42 were added to the treatment solution on D0; therefore, these data served as references to the upcoming ones. For both rotifer species, organ shrinkage was less pronounced on D20 than in adequately untreated controls. In the Aβ42-treated groups, significantly greater regeneration was observed on D25 than in the other D25 groups. These measurements indicate that the aggregate with a natural structure also has a specific modulating effect. Furthermore, organ shrinkage was less in the Aβ42-treated groups than in the S-Aβ42 counterparts. The same phenomenon was also observed at the end of regeneration when the animals

recovered significantly in the presence of Aβ42. These data showed that the attenuation of shrinkage via modulation is likely sequence-specific, since the order of amino acid is the only difference between the two types of Aβs. On D20, the ConA inhibited the FROS in rotifer species in a food-free but glucose-containing environment.

4.2.2. Depletion

Four species were treated with Aβ42 aggregates; only two secreted Rotimer (*E. dilatata*; *L. bulla*). Rotimer depletion was used in exudating animals to investigate the role of this biopolymer in Aβ sensitivity and toxicity. We found that *E. dilatata* and *L. bulla* are not sensitive to Aβ42 aggregates in their healthy state, where the esterase activity and the number of living rotifers are the same as those of the species-specific untreated controls (100%). After *in vivo* biopolymer depletion, the animals lost their resistance against neurotoxic aggregates; the measured parameters were significantly reduced compared to their controls. A similar phenomenon (significant decrease) was also observed in those animals (*S. pectinata; C. intuta*) that did not secrete exogenous biopolymer.

5. Discussion

Rotifers, as micrometazoans, are widely accepted ecological indicators and experimental animal models of aging, lifespan, toxicology, space exploration and pharmacology research. Bdelloid rotifers (e.g., *P. acuticornis*) can catabolize neurotoxic aggregates *in vivo*.

Our team has recently discovered and published the fact for the first time that rotifers are also capable of producing special biopolymers. Due to its novelty, this family of rotifer-specific biopolymers, namely 'Rotimers', is not yet known in detail. Their product is the Rotimer-Inductor Conglomerate (RIC). In addition, it is significantly modified according to physiological-, drug-and environmental effects. This filamentous, film-, and glue-like viscoelastic secretion has been observed in several monogonant and bdelloid species and are used by these small creatures to clean the medium (antiseptic and filtration effect), from food capture to egg placement (adhesive property).

Furthermore, we were also interested in how RIC can maintain its integrity in the presence of various chemicals. Various environmental factors (e.g., temperature, pH, metal ions, and chemicals) affect the production of Rotimer. The fact that our subjects retained this ability confirms the idea that this product is a multifunctional bioactive substance that also has a significant role as a bioindicator. Extensive investigation of Rotimer has many possibilities for various practical applications in the future.

During the bioactivity experiments, this exudate has motility-inhibition effects in different cell types (e.g., algae and yeast). The measurements have shown their diverse and promising bioactivity inhibition of cancer neuroblastoma cell proliferation. The interaction between RIC and aggregates shows that *E. dilatata*- and *L. bulla*-RIC, with proteinous exudate on their surface, specifically bind the H-Aβ, which is an efficient influencing factor in aggregation kinetics;

moreover, the activity of the species-specific biopolymers may differ from each other. There could be more explanation of the different behavior of the RIC on H-Aβ-binding and aggregating processes, derived from the two different species. One interpretation could be that *L. bulla* originally produced and adhered less biopolymer to the epoxy beads than *E. dilatata*. Another possibility could be that the biopolymers may differ in the various rotifer species in structure, functionality, and efficacy. The anti-aggregation effect of LB-RIC was not increased when the number of animals was higher with longer Rotimer secretion time, or a two-fold dose of RIC was tested in the presence of standard H-Aβ amount. The interaction between the Rotimer and the neurotoxic peptide aggregates (e.g., alpha-synuclein and prions) needs further investigations. It is well-known that during the Rotimer secretion the calcium is built-in and it may provide extra ionic characteristic to this biomaterial, therefore the metal ion content of biopolymer may enhance these molecular relations. The mechanism of anti- and disaggregating ability could be like the action of beta-sheet-breaker molecules or that of some natural peptide sequences. In summary, it is important to highlight that the direct molecular effects of natural polymers on neurodegeneration-related amyloids have been barely investigated, but there are just a few cases. Based on the results obtained, it is assumed that Rotimer has some breaker activity, which can dissociate H-Aβ aggregates. The rotifer-specific biomaterials, namely Rotimer, were used for that purpose for the first time. Indirect *in vivo* measurements have been done with respect to the protective role of Rotimer against H-Aβ, but *in vitro* binding, anti- and disaggregating experiments have never been performed previously. Moreover, this interdisciplinary approach may open novel perspectives also in pharmacological research against Alzheimer's disease.

Although this proteinous biopolymer's molecular structure and holistic biotechnological and biochemical significance are not yet known, further physicochemical analyses will be required. Based on the universal adhesive property of Rotimer, it may prove to be a useful tool in wastewater cleaning. Similarly, to other biopolymers, it may have a potential industrial use.

Before, nobody has investigated the *in vivo* catabolism of the Aβ as a food source or potential autocatabolism-regulator for multicellular entities. We applied an organ size-based *in vivo* monitoring system, exploring the autocatabolism-related alterations evoked by Aβ42, in a glucose-supplemented starvation model. The empirically well-monitored reduction in the size of bilateral germovitellaria (reproductive organ) in starved rotifers was attenuated by Aβ42, which served as a nutrient source- and peptide sequence-specific moderator during the organ shrinkage phase and enhanced the regenerative phase, including egg reproduction. The starvation-induced shrinkage of germovitellaria, with their regeneration and reproduction capability in these animals, is an adequate physiologic and experiential marker of autocatabolism, summarized by $FROS_i$. The FROSi of natural aggregates, in contrast to their artificial scrambled version, demonstrated that the Aβ42 is not only a food source for rotifers but also a potential regulator and moderator of their systemic

metabolism. *Philodina* and *Adineta* species showed similar types of lesions; therefore, it can be stated that these effects of Aβ42 are not limited to a specific species. By applying these microscopic invertebrates, the hitherto unknown roles of Aβ42 were demonstrated, providing additional tools for exploring relations between neurotoxic aggregates and metabolism. In another experiment, after *in vivo* biopolymer-depletion, the animals lost their resistance against neurotoxic aggregates. The above results suggest that Rotimer may play an essential protective role against Aβ aggregate toxicity in rotifers.

The cosmopolitan rotifers occur in fresh or saline water, soil, and arctic/high-altitude ice sheets. Their bioactive exudate can also play a globally decisive ecological regulatory and executive function. The impact of rotifers on the environment seems surprisingly wide-ranging. Based on this fact, we aimed to understand the mechanism of Rotimer production, their anatomical, physiological, genomic, and proteomic background, as well as their evolutionary biodiversity, their ecological role in nature, and their interdisciplinary translation possibilities. To have further and long-term plans for this natural organic product, comprehensive chemical and biological exploration is inevitable and necessary. Biopolymers can be used in all scientific fields, from nature conservation technologies to industrial nutrient production, the delivery of genes and drugs, or the production of nanoparticles to tissue regeneration. Our preliminary studies with Rotimers have not by far exhausted the versatility of this newly discovered and published molecule family, the natural and innovative potentials inherent in them.

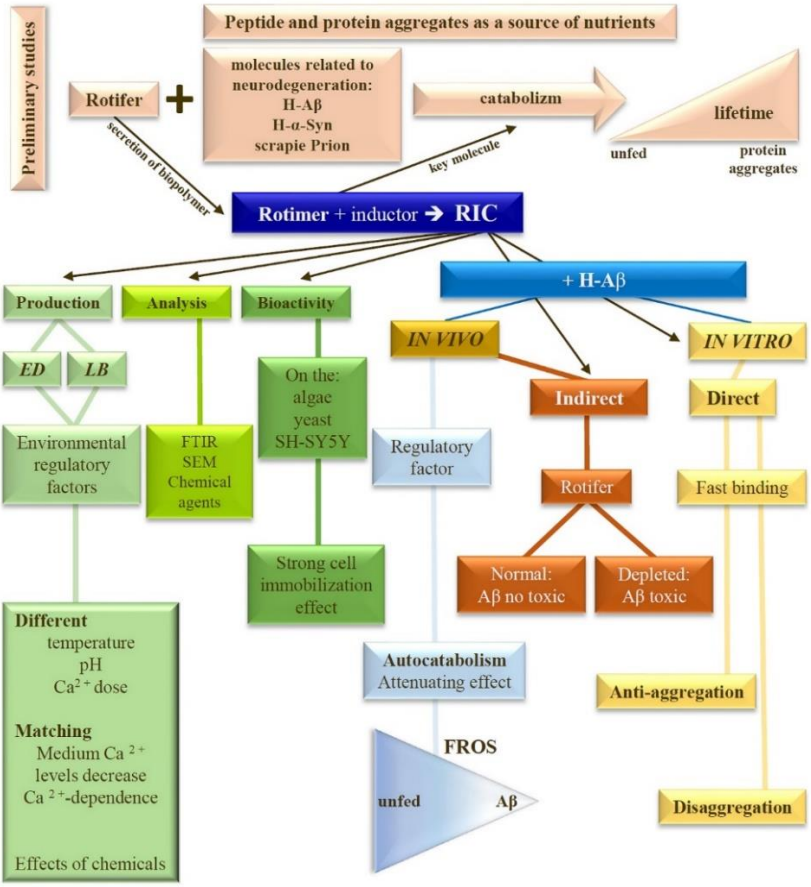
6. Conclusion

The result of our work describes a novel and bioactive rotifer-specific biopolymer, called Rotimer, derived from certain monogonants. The motility-inhibiting effect of this bio-exudate was observed in various cell types, and its protective effect against dementia-specific aggregates in rotifers. During the investigations, it was found that the production of Rotimer proved to be obligatorily calcium-dependent; moreover, this special exudate is a promising bioindicator of the natural aquatic environment.

Our research group was the first to describe the in vitro interaction between Rotimer and H-Aβ by presenting biopolymers' anti- and disaggregating effects against Alzheimer's disease-type Aβs. The obtained results reflect the relationship between RIC and H-Aβ, in addition, the stable interaction thus formed also shows Aβ sequence specificity, since there was minimal binding between RIC and S-Aβ. The conglomerate of both monogonant rotifer species (*E. dilatata and L. bulla)* molecularly interacted with H-Aβ considered oligomer or fibril type; moreover, the ED-RIC was more effective in connecting and aggregation processes. Due to its properties mentioned above this natural material needs to study in the future; moreover, this biopolymer can be a relevant molecular specimen for developing drug candidates in neurodegeneration-related research.

Summary of the main findings were the following:

1. An exceptional relationship can develop between rotifers and human-type beta-amyloid (Aβ) aggregates under laboratory conditions (e.g., using amyloid as a nutrient source).
2. As an exudate production, the secretion of rotifer-specific biopolymer (Rotimer) is an obligatory calcium-dependent process and a philogenetically ancient phenomenon in rotifers.
3. Rotimer is a proteinous biomolecule that is sensitive to proteases.
4. Rotimer is not a toxic substance for different biological models but can universally block the motility of different cell types.
5. Endogenous Rotimer has a protective effect *in vivo* against the toxicity of Aβ aggregates.
6. Aβ aggregates are not only sources of nutrients but also regulators of the rotifer's metabolism.
7. Rotimer specifically binds to Aβ aggregates *in vitro* and affects their aggregation.
8. Rotimer may contain promising molecular information for Alzheimer's disease drug discovery.



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