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REVIEW

Yeast red pigment, protein aggregates, and amyloidoses: a review

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Abstract
Estimating the amyloid level in yeast *Saccharomyces*, we found out that the red pigment (product of polymerization of aminoimidazole ribotide) accumulating in *ade1* and *ade2* mutants leads to drop of the amyloid content. We demonstrated in vitro that fibrils of several proteins grown in the presence of the red pigment stop formation at the protofibril stage and form stable aggregates due to coalescence. Also, the red pigment inhibits reactive oxygen species accumulation in cells. This observation suggests that red pigment is involved in oxidative stress response. We developed an approach to identify the proteins whose aggregation state depends on prion (amyloid) or red pigment presence. These sets of proteins overlap and in both cases involve many different chaperones. Red pigment binds amyloids and is supposed to prevent chaperone-mediated prion propagation. An original yeast-*Drosophila* model was offered to estimate the red pigment effect on human proteins involved in neurodegeneration. As yeast cells are a natural feed of *Drosophila*, we could compare the data on transgenic flies fed on red and white yeast cells. Red pigment inhibits aggregation of human Amyloid beta and α-synuclein expressed in yeast cells. In the brain of transgenic flies, the red pigment diminishes amyloid beta level and the area of neurodegeneration. An improvement in memory and viability accompanied these changes. In transgenic flies expressing human α-synuclein, the pigment leads to a decreased death rate of dopaminergic neurons and improves mobility. The obtained results demonstrate yeast red pigment potential for the treatment of neurodegenerative diseases.

Keywords Amyloid · Prion · Yeast red pigment · Neurodegeneration

Introduction

Though protein quality control (Bukau et al. 2006; Alberti 2012) fights misfolding and aggregation, they remain a considerable challenge for living cells and consequently a substantial problem of cell biology and medicine. Numerous conformational diseases (Needham et al. 2019) are caused by protein conversion to amyloid state. Amyloids are protein aggregates characterized by a fibrillar morphology, beta-sheet secondary structure, and ability to be stained by specific dyes, such as Thioflavin T (ThT) or Congo red (Sipe et al. 2016). In some cases, the amyloid state can be transmitted vertically (from parents to progeny) and horizontally (from cell to cell or organism to organism). Such inheritable infectious amyloids are called prions (Prusiner 1998).

Our present-day progress in understanding prions is connected with studying of these proteins in the *Saccharomyces cerevisiae* yeast. There are several causes for this. This eukaryotic microorganism is a convenient object of powerful genetic and molecular biological approaches. Mechanisms of apoptosis, autophagy, and protein quality control relevant for conformational diseases are highly conserved and apply for yeast (Botstein and Fink 2011; Andrews et al. 2016). Several yeast prions are well-studied. First of them were described more than 25 years ago by Wickner (1994; for authoritative reviews, see Wickner et al. 2018 and Chernoff et al. 2020). In many cases, prion state does not lead to drastic effects on yeast viability. Even more, prion [PSI+] cause genome wide readthrough translation that sometimes increases evolvability in certain harsh environments (Lancaster et al. 2010). Though there are contrasting data, for examples, a drastic "suicide" Psi mutation (McGlinchey et al. 2011).

And at last, chimerical constructs employing human amyloidogenic proteins fused to fluorophores and expression vectors allow for effective analysis of disease-related protein aggregation in yeast cells (Tuite 2019).

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Amyloids are self-propagating. They can recruit monomers of the protein and convert them into fibrous cross-beta aggregates via a templated nucleated polymerization. Prions have a particular position among amyloids, as they are able to support intensive self-propagating amyloid state in successive generations due to the assistance of chaperones. Without being amyloids, chaperones affect the folding and are involved in generation of oligomeric "seeds" (sometimes called "propagons") — that pass to daughter cells and serve as templates in amyloid self-propagation (Chernoff 2004; Cox et al. 2007; Kushnirov et al. 2007; Rikhvanov et al. 2007; Wickner et al. 2007, 2008).

The first chaperone with a firmly established role in prion propagation was Hsp104p. It functions by breaking up large prion aggregates for resolubilization, producing seeds that are transmissible to daughter cells during cell division (Chernoff et al. 1995; Kryndushkin et al. 2003). Almost all identified yeast prions require Hsp104 for propagation. At the same time, they differ in their response to Hsp104 overproduction. Prion Sup35 is most efficiently cured by surplus Hsp104 (Chernova et al. 2014, 2017).

Ca. 10 different amyloid-based prions are described for *Saccharomyces* yeast, and the function of their normal (non-prionized) form is determined (Chernoff et al. 2020). The most well-studied prion Sup35p, named also [*PSI*$^+$], is one of the translation termination factors, eRF3. Its inactivation due to prionization leads to nonsense suppression. In our studies, the [*PSI*$^+$] factor was recognized by suppression of an *ade1* and *ade2* nonsense mutations. These mutations lead to the blockage of adenine biosynthesis and accumulation of red pigment (RP). Suppression of these mutations results in reduced amount of RP and growth in a synthetic medium without adenine. The visual express test allows us to follow the changes in [*PSI*$^+$] content.

We screened for proteins whose aggregation depended on prion presence. Such prion-dependent proteins can provide new ways for studying prion life cycle. We learned that RP accumulation in yeast *ade1* and *ade2* mutants affects prion propagation and toxicity. We have also shown that RP affects the expression of human amyloidogenic proteins such as Abeta and α-synuclein. To further study RP effect on these amyloid proteins involved in neurodegeneration, we used *Drosophila* model. Transgenic strains of this organism are relatively easily obtained and cultivated; they have a short life span. Old fruit flies' neurons can be damaged by neurodegenerative disease amyloids (Marsh and Michels 2006; Bolus et al. 2020).

Identification of [PSI+]-dependent proteins

In the paper by (Nevzglyadova et al. 2008), we studied the effect of [*PSI*$^+$] on composition of the pellet obtained by cell lysates spinning (Fig. 1). We got pairs of isogenic [*PSI*$^+$] and [*psi*−] strains by different prionotropic treatments: elimination of [*PSI*$^+$] and possibly some other prions by

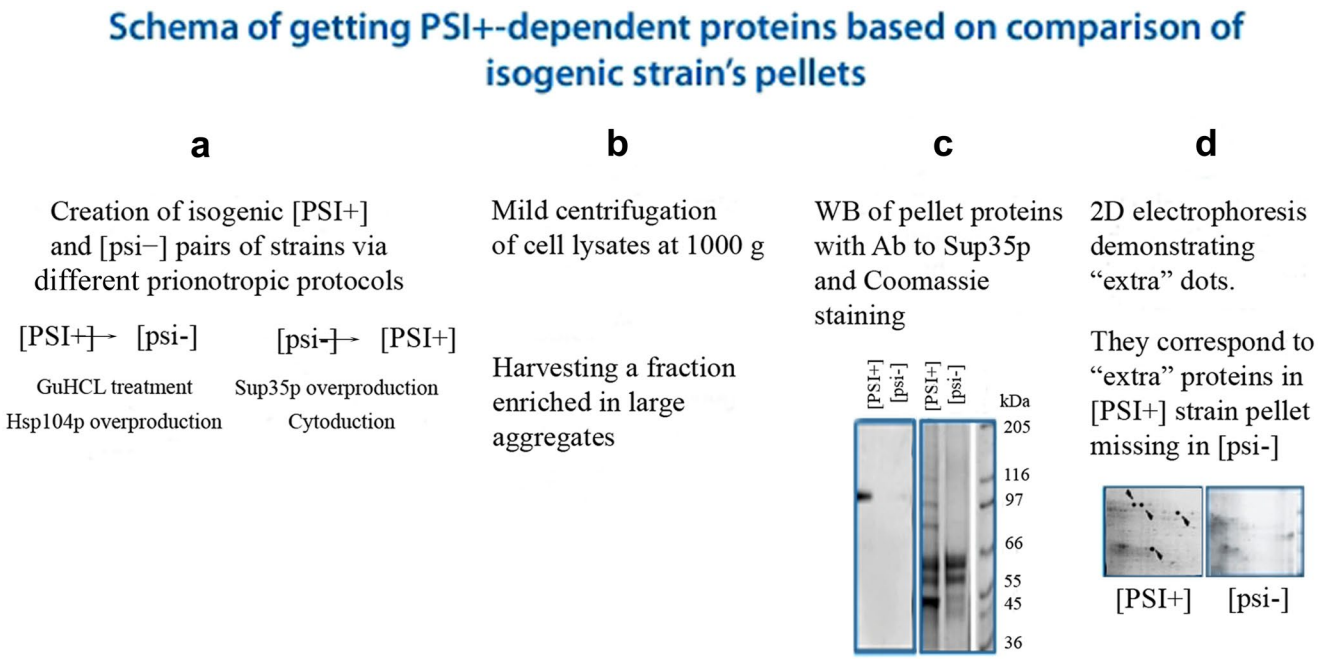

Fig. 1 Scheme of comparative study of [PSI+] and [*psi*$^-$] isogenic strains and search for [PSI+]-dependent proteins

GuHCl; elimination of [*PSI*$^+$] by overexpression of chaperone *HSP104* gene carried on a plasmid; and augmentation of [*PSI*$^+$] factor by overexpression of the *SUP35* gene and transmission of [*PSI*$^+$] factor by cytoduction (Fig. 1a). In further experiments the electrophoretic patterns and Western blots of pellet proteins in the [*PSI*$^+$] and [*psi*−] isogenic strains were compared (Fig. 1b, c). We found not only predictable differences in the presence of Sup35p but also detected bands not related to Sup35p whose presence or amount was dependent on the [*PSI*$^+$] state. We used 2D electrophoresis to identify the pellet proteins that can be lost or depleted in [*psi*−] cells (Fig. 1d). Our techniques focused on delicate procedures of harvesting the pellet (aggregation) fraction of cell lysates and "genetic purification," meaning comparison of isogenic strains differing only by their prion content.

A consequent MALDI analysis allowed us to identify the proteins present only in [*PSI*$^+$] partners of the strain pairs. A large group of ca. 40 prion-associated proteins were identified. Approximately half of these proteins belonged to chaperones and enzymes of glucose metabolism. Chaperones were known to be involved in prion metabolism and so are expected to be present in prion-containing aggregates, but glucose metabolism enzymes were not predicted to be present. Also, we detected proteins involved in oxidative stress response and translation.

We proposed dividing the prion-dependent fraction of pellet proteins into two classes: (a) the "target" proteins, such as prions, stress damaged (misfolded) proteins, and some oxidatively impaired glucose metabolism enzymes. They undergo specific manipulations intended to mend, disintegrate, or neutralize them. And (b), the "tool" proteins that carry out these manipulations, such as chaperones and other proteins involved in posttranslational modification of proteins, like Sec53p and proteinases. In some cases, this classification is likely to be an oversimplification; we know that Eno2p is both a glucose metabolism enzyme and a co-chaperone.

During performing this study (Nevzglyadova et al. 2009, 2010a), several laboratories identified and characterized proteins associated with purified aggregates containing a [*PSI*$^+$] prion (Bagriantsev et al. 2008) or a prion-related polyglutamine domain of huntingtin exon 1, cloned in yeast (Wang et al. 2007, 2009). Erjavec et al. (2007) studied carbonylated proteins associated with Hsp104p-containing protein aggregates. The data obtained in these works were interpreted mainly using an aggresome concept. The mammalian perinuclear aggregate of misfolded proteins was labeled as aggresome (Johnston et al. 1998; Kopito 2000). Chaperone action, stress response, and proteolysis are also closely related to the aggresome functions. During the following few years, several other aggregate deposits of yeast cells were described diligently. Those comprise peripheral CytoQ (Miller et al. 2015), JUxtaNuclear Quality Control Compartment (JUNQ) (Kaganovich et al. 2008); IntraNuclear Quality Control Compartment (INQ) (Gallina et al. 2015; Miller et al. 2015); and Insoluble PrOtein Deposit (IPOD) (Kaganovich et al. 2008; Rothe et al. 2018). CytoQ, JUNQ, and INQ host predominantly misfolded cytosolic and nuclear proteins that are relatively soluble and exchange rapidly with the surrounding cellular material, whereas the IPOD seems to harbor predominantly amyloids and other less soluble, terminally aggregated misfolded proteins. Protein deposition in CytoQ and IPOD is mediated by Hsp42 chaperone (Specht et al. 2011; Grousl et al. 2018).

RP modifies amyloid content

Studying amyloid content in many different strains, we unexpectedly found that the intensity of amyloid-bound ThT fluorescence (IF ThT) depended not only on the lysate fraction and prion status but also on the color of the culture. Strains with *ade1* and *ade2* mutations that accumulated RP manifested lower IFs than "white" isogenic strains (Nevzglyadova et al. 2010b). (In denoting cells', mutants', and cultures' color, we do not use quotes as they are really colored; we use them referring to strains.)

We measured the IF and mobility of pellet proteins in agarose gel in mutant *ade1* and *ade2* cultures grown in a medium with a tenfold higher concentration of adenine (YEPD-ADE), which prevents the accumulation of RP. When cells were grown on a YEPD-ADE, their IF ThT significantly increased, while in strains prototrophic for adenine (i.e., not carrying any mutations in adenine biosynthesis genes), IF ThT values are high and independent of the adenine amount in the medium.

Using UV mutagenesis, we induced secondary mutations that suppressed the accumulation of RP in mutant *ade1* and *ade2* strains. In these "white" mutants, IF values turned out to be significantly enhanced. On the other hand in "white" Ade$^+$ strains, we induced mutations which led to the accumulation of RP accompanied by a considerable drop of IF ThT values. The results for ThT IF values of pellet proteins isolated from "red" and "white" isogenic strains are in good agreement with the agarose electrophoresis data. Enhanced IF is followed by an increased fraction of high MW proteins. In experiments with independent [*PSI*$^+$] "red"/ "white" strain pairs using blot-hybridization with Sup35p antibody, we demonstrated that the amount of aggregated Sup35p is higher in "white" strains.

RP prolongs life span in yeast

Based on our data (Nevzglyadova et al. 2010b, 2011; Mikhailova et al. 2011), we can conclude that yeast RP affected amyloid life cycle. Although some therapies for several proteinopathies have been proposed recently, the search for effective drugs is not over (Bayer 2015; Bitan 2019). We believe that yeast (which human beings have been consuming for thousands of years) may be a potential source for the development of harmless anti-amyloid compounds. The therapeutic application of RP depends on a crucial point — the RP's effect on cell viability. In our experiments, several pairs of isogenic strains grown for stationary phase on a synthetic complete medium were compared. In each pair, both strains carried a mutation in either *ADE1* or *ADE2* genes, but only one partner was "red" due to accumulation of the RP, while the other was "white" due to an additional mutation in any of the upstream purine biosynthesis genes (Nevzglyadova et al. 2011). We learned that in each "red"/ "white" pair of strains, the "red" cells contained fewer amyloids and had a better survival rate.

Identification of RP-depending proteins

Figure 2 presents a comparison of "red"/ "white" strain pairs in [*psi*⁻] background. The results speak in favor of RP capacity to diminish amyloid amount. It was interesting to learn whether the pellet fraction of white cells contains proteins not present in pellets of isogenic red cells. The pellet proteins isolated from 3 independent "red" and "white" [*psi*⁻] pairs were compared by 2D electrophoresis. The data showed that the "white" strain really contained some extra proteins. It means that corresponding proteins in "red" strain most likely lack some components of aggregation machinery. In our case as suggested by ThT data, it can be amyloid.

Twenty-three extra (RP-dependent) proteins were identified by MALDI analysis in pellets of "white" strains. These proteins seem to belong to a general class of amyloid-dependent proteins. Their association with amyloids leads to aggregation and hence sedimentation in the pellet fraction. As RP diminishes the amount of amyloids, it is supposed that the total amount of pellets will also drop. This effect may be enhanced by competition between RP and the chaperones for the sites on amyloid molecules. The ability of RP to compete with ThT for binding with amyloid fibrils was shown earlier (Mikhailova et al. 2011). It allowed us to suggest that sites required for binding of chaperons or other prion-dependent proteins may be occupied by RP, and this consequently will lead to the drop of pellet content.

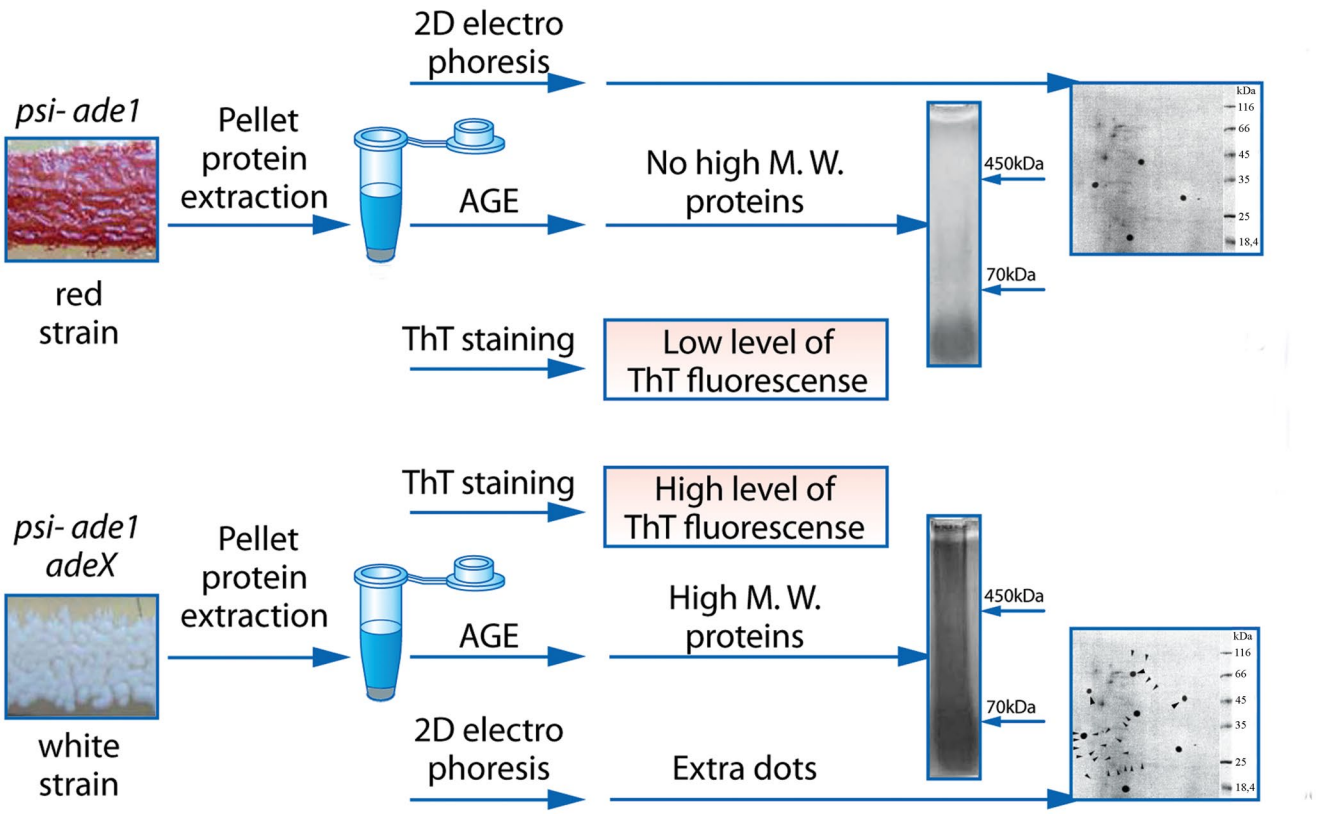

Fig. 2 Comparison of pellet proteins of [*psi*⁻] red/white isogenic strains and search for pigment-dependent proteins

Figure 3 manifests sum data on identification of prion and pigment-dependent proteins. In both cases, we are based on the differences in pellet fractions though it must be noted that prion-dependent proteins are "prion-philic" when RP-dependent proteins are "pigment-phobic."

In total, our 2D electrophoresis-MALDI experiments allowed us to identify 84 pellet proteins — among them 19 different chaperones, 20 stress response proteins, and 6 proteins involved in proteolysis (Nevzglyadova et al. 2012). Our list of identified prion- and RP-dependent proteins is closely related to the lists of proteins identified in yeast aggregates involved in protein quality control identified by other authors (Wang et al. 2007; Bagriantsev et al. 2008; Erjavec et al. 2007). Correspondence with the list of proteins mobilized to yeast aggresomes (Wang et al. 2009) is unlikely to be accidental, as Cdc48p, one of the crucial proteins of aggresomes, was also found among the RP-dependent proteins.

An age-related degenerative disease ALS (amyotrophic multiple sclerosis) involves the accumulation of aggregated superoxide dismutase of *SOD1* with an amyloid-like structure, and the aggregates were shown to propagate in a prion-like manner in neuronal cells (Elam et al. 2003; Banci et al. 2008; Munch et al. 2011; Estácio et al. 2015; Ayers et al. 2016; Shvil et al. 2018; Malik and Wiedau 2020). In our studies, the copper-zinc superoxide dismutase (Sod1p) was identified as an RP-dependent protein, and its mitochondrial isoform manganese SOD (Sod2p) was identified as a prion-dependent protein. In the most promising paper, Bondarev et al. (2018) reviewed numerous recent studies covering co-aggregation triggered by amyloids.

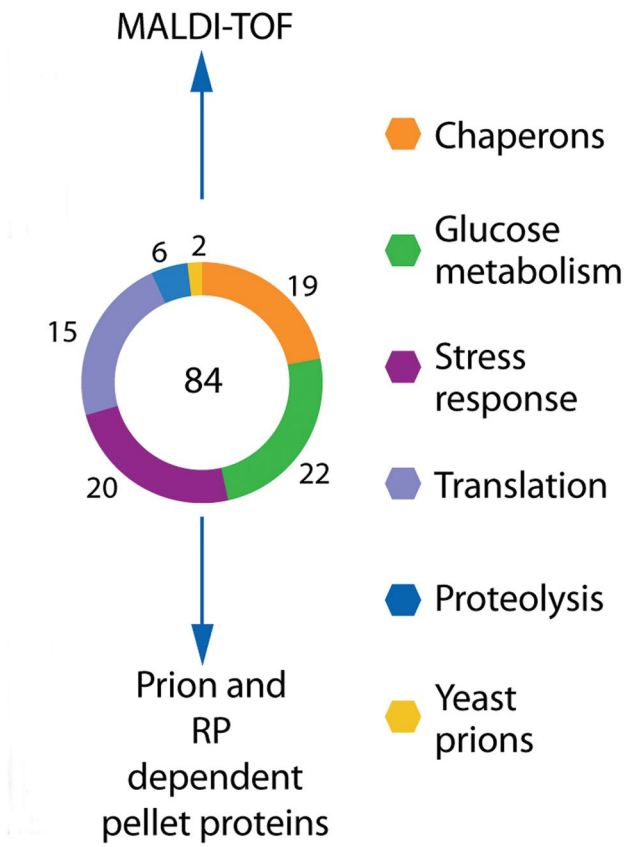

Fig. 3 Prion and pigment-dependent proteins identified in our studies

RP structure

RP of yeast *Saccharomyces cerevisiae* belongs to the group of N-heterocyclic compounds that include purines, pterines, and their derivatives. Cultivation under aerobic conditions of the yeast *Saccharomyces cerevisiae* with mutated gene *ADE1* or *ADE2* leads to accumulation in the cell cytoplasm of 5-aminoimidazolribotyl (AIR). It is believed that this compound is transported (glutathione-dependently) into vacuoles, where it is converted into the RP, as a result of polymerization and modifications (Smirnov et al. 1967; Fisher 1969; Sharma et al. 2003). RP accumulation ensures the red color of the mutant colonies. The RP can be involved in interesting physiological effects, such as DNA cleavage modification (Meskauskas et al. 1985). The structure of the RP has not yet been established; however, it has been shown that it includes imidazole, ribose, and amino acids and does not contain phosphates.

We extracted the RP according to a modified protocol (Amen et al. 2013) and studied the chemical structure of the polymer by MALDI-TOF analysis. Apart from the natural RP form, we obtained ribose-free RP acidic hydrolysate and created a synthetic methylated compound not containing ribose and amino acids (MeAI). We learned that natural RP comprises a set of polymers with molecular mass from 2 to 10 kDa, whereas hydrolyzed form has a significantly lower molecular mass (on average, 1–2 kDa). However, we have failed to reveal indications for the regular structure of these polymers. This irregularity is most likely due to different amino acids attached to different monomers. This suggestion is supported by mass-spectrometry analysis of synthetic polymer MeAI containing no amino acids. MALDI analysis of MeAI revealed peaks corresponding to 4, 6, 7, 8, 9, and 10 structural units (Fig. 4a). Figure 4b presents a hypothetical schema of synthesis (oxidative polymerization) of MeAI. Predicted molecular masses of polymers are closely followed by the data on MALDI-TOF. Differences are negligible.

RP effect on insulin and lysozyme fibril growth

We have also studied how the binding of RP affected the formation of insulin and lysozyme fibrils in vitro. Data of electron microscopy (TEM) demonstrate RP effect on growth of protein fibrils (Fig. 5a, b). Since fibrils stuck together into large aggregates, we sonicated them for 1 min using a routine sufficient for fibrils untreated by RP. It turned out that the aggregates formed

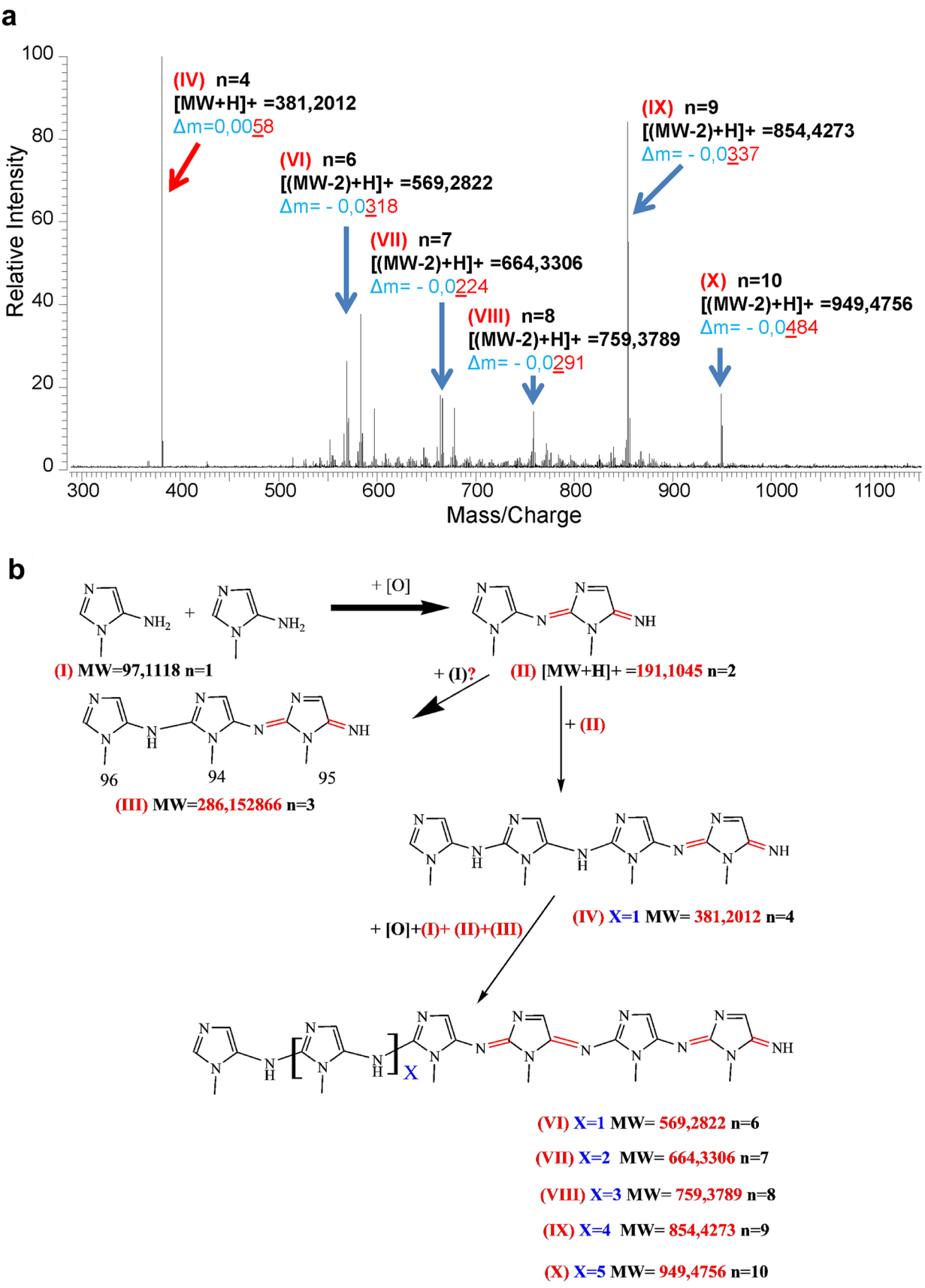

Fig. 4 Synthetic red pigment (MeAIR): MALDI-TOF **a** and synthesis pathway **b**

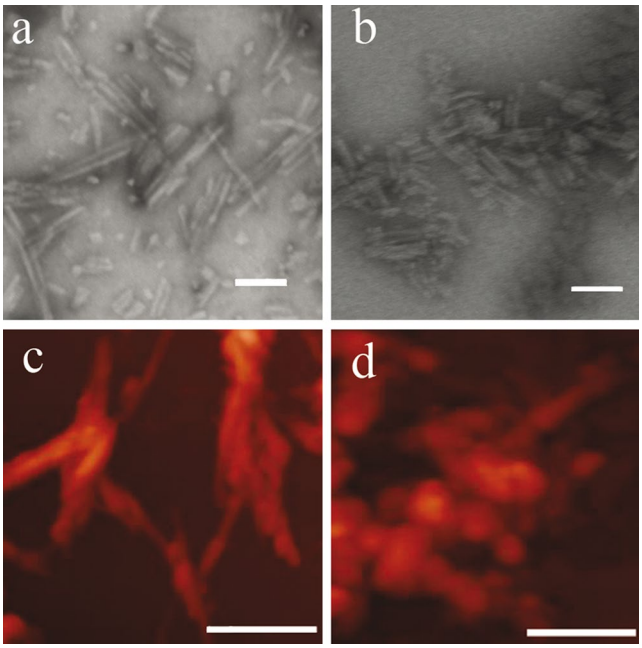

Fig. 5 TEM and AFM images of lysozyme fibrils grown in absence **a**, **c** and presence **b**, **d** of red pigment. **a** Oligomers and mature fibrils, **b** coalescent short fragments of fibrils, **c** single fibrils and bundles, **d** coalescent short fragments of fibrils. Scale 100 nm **a**, **b** and 200 nm **c**, **d**

in the presence of RP were not disaggregated. When ultrasound treatment was intensified fourfold, control fibrils were not merely separated but disaggregated into individual small fragments. At the same time, the fibrils grown in the presence of RP stood as large aggregates. It can be suggested that RP causes coalescence of insulin and lysozyme fibrils, leading to increased stability of aggregates. Data of atomic force microscopy (AFM) (Fig. 5c, d) confirmed the results of electron microscopy. Whereas in the control amyloid preparation, both individual fibrils and parallel fibril bundles are discernible; in amyloid grown in the presence of RP, linear structures were practically not seen.

A similar effect of fibril coalescence was also shown by other authors. AFM of collagen amyloid grown in solution of GuHCL revealed protofibril coalescence (Lancaster et al. 2010). With the aid of atomic-force microscopy and spectroscopy, Smith et al. (2006) analyzed the kinetics of growth of individual insulin fibrils. These authors demonstrated that recruitment of monomers into the self-organizing polymer chains requires free ends formed spontaneously, as a result of disruptions of fibrils reaching a definite length. Taking into account these data, it can be inferred that anti-amyloid activity of RP is due to reducing the number of free ends or making them less accessible for attachment of new monomers, or both.

RP effect on Abeta expression in yeast/fruit fly model and also in vitro

Alzheimer's disease (AD) is a most frequent neurodegenerative late age malady distinguished by progressive cognitive and behavioral changes and memory impairment (Tiwari et al. 2019). AD pathology is characterized by depositions of extracellular amyloid plaques, intracellular neurofibrillary tangles, and neuronal loss. Amyloid plaques are composed of insoluble 39–43 amino acid peptides named the amyloid beta (Abeta). Abeta is produced in the extracellular space as a result of the proteolytic cleavage of the transmembrane amyloid precursor protein (APP) by beta- and gamma-secretases (Glenner and Wong 1984; Iwatsubo et al. 1994; Lane et al. 2018). Different steps of Abeta amyloid assembly include the formation of several metastable structures, such as monomers, small linear oligomers and their aggregates, spherical oligomers, and protofibrils, which might be components of a pathway to fibril formation (Selkoe 2008). It was shown that the impact of Abeta oligomers on patients' brains is even more severe than that of Abeta plaques (Benilova et al. 2012; Guo et al. 2020). At the same time, the soluble Abeta monomers are normal components of the human brain and exhibit protective functions (Copani 2017). They serve as a positive endogenous regulator of synaptic vesicle release also promoting autophagy and inhibiting apoptosis caused by oxidative stress.

Several lines of evidence suggest that the process of amyloid formation plays a central role in the AD pathogenic pathway (Chen and Mobley 2019). Therefore, one of the approaches to the anti-amyloid therapy is the production of beta- and gamma-secretase inhibitors (Koseoglu 2019). However, all well-known inhibitors of both secretases are very toxic and have different side effects (Kukar et al. 2007; Laras et al. 2009). All the more, the evidence for detailed mechanisms of neuron death is far from clear (Mukhin et al. 2017). AD pathogenesis is considered to depend not only on Abeta oligomers but also on Abeta oligomers integrated at the cell surface into complexes with other proteins (like PrP^c and Glu R5). So it was found actually to search for drugs that can improve the catabolism, sequestration, and clearance of Abeta and block its aggregation (Townsend et al. 2006; Meldolesi 2019).

In our experiments, we studied how RP modifies Abeta aggregation in AD models of both *S. cerevisiae* and *Drosophila melanogaster* (Nevzglyadova et al. 2015). A multicopy plasmid carrying a human Abeta-42 coding sequence (Irie et al. 2005) fused to the green fluorescent protein (GFP)-coding gene under CUP1 promoter control (Rubel et al. 2008; Saifitdinova et al. 2010) was used for the

transformation of "red" and "white" yeast strains. The "red" strain carrying *ade2* mutation accumulated RP, while the "white" one with an additional mutation *ade4* did not. The *ade2* transformants exhibited different colony colors after 2–3 days of incubation on media at different adenine contents: at low concentration, they were red; at medium, they were pink; and at high, they were white. We learned that increased adenine concentration led to reduced RP accumulation and at the same time induced an increased number of Abeta-GFP aggregates (cytoplasmic inclusions or foci). The analogous comparisons with "white" (*ade2 ade4*) transformants were not statistically significant. The pronounced effect of high adenine concentration on protein aggregation in *ade2* cells and the absence of statistically significant effects in *ade2 ade4* cells suggest that the formation of fluorescent foci is inhibited via RP.

Another specific effect of RP on Abeta level was estimated by Western blot analysis of pellet proteins treated with Abeta antibodies. Comparison of 18-h-old transformant cells grown and harvested in the logarithmic phase demonstrated that the amount of Abeta in pellets did not vary with different adenine concentrations. By contrast, 4-day old cells in the stationary phase contained considerably higher amounts of hybrid protein in the pellet at higher adenine concentration. We also used A11 antibodies reacting with amyloid oligomers. A11-positive staining was abundant in the "white" strain but decreased in the "pink" one and was very low in the "red" one. Western blot analyses of yeast cell lysate pellets stained with A11 antibodies were given special attention. Several authors have shown that small oligomers of different amyloids form similar conformational structures (generic epitopes) that can be targeted by the A11 antibodies (Kayed and Glabe 2006). A11-positive oligomers were shown to be highly toxic. A11-positive plaques were found in the brains of AD patients. Soluble Abeta oligomers are considered to be promising targets for AD therapy (Hefti et al. 2013). We found that the presence of RP led to a decreased amount of A11-positive oligomers. This drop in the amount of soluble Abeta oligomers can turn up to be of therapeutic value.

All the above-mentioned results were obtained with yeast cells differing in their ability to accumulate endogenous RP. Exogenous RP allows minimizing some possible pleiotropic side effects in living cells. Pigmentless plasmid lacking (pl^-) cells were used to study RP penetration which was registered by the RP own fluorescence level. Most pl^- cells grown at low adenine concentrations synthesized fluorescing RP that accumulates in vacuoles. Cells grown at the high concentration were dark, but if the RP was added to the medium, they began to fluoresce. The fluorescence was often clearly localized in vacuoles and was not removed by washing. The Western blot data on pellet proteins confirm that the amount of Abeta-GFP aggregates in "white" cells reduces when exogenous RP is applied.

The RP influence on the growth of Abeta aggregates was also studied in vitro. We grew Abeta fibrils in the presence of RP, while in control experiments, they were grown in the same buffer without RP (Gonzalez-Velasquez and Moss 2008).The growth of fibrils was estimated by two methods. The optical density increase at 365 nm was measured spectrophotometrically, and amyloid bound ThT fluorescence was registered. The decrease in RP-dependent Abeta growth was estimated by both methods. The effect of the RP on Abeta growth was visualized by TEM. Based on the obtained results, we propose that RP inhibits amyloid growth during the early stages of fibril formation (when globular oligomers and protofibrils are abundant).

Transgenic *Drosophila melanogaster* expressing the human Abeta42 peptide in the neurons was used as another model system to study the RP effect on AD. The transgenic flies were fed on isogenic "red" and "white" yeast strains from eclosion until death. The resulting "red" and "white" flies were compared. Amyloid content was estimated by the fluorescence intensity of amyloid-bound ThT of the homogenized flies on the 14th and 21st days. The decrease in amyloid content in Abeta42 "red" compared to "white" flies was most pronounced in the 21-day old flies group.

Abeta42 level in the head lysates of flies consuming red and white yeast was compared by Western blot. The Abeta42 level normalized to protein of flies consuming red yeast was lower in both soluble and insoluble fractions. The neurodegeneration area in flies fed on red yeast was much more reduced than in the flies fed on white yeast, especially in 30-day old flies. The effects of these changes were accompanied by improved climbing behavior. The RP effect on learning and memory in flies was also investigated in old flies. The index of learning and memory was 2- to threefold higher in Abeta42 flies consuming red vs. white yeast. In contrast, the flies lacking Abeta consuming red or white yeast did not demonstrate any statistically significant difference. The data obtained from experiments on *Drosophila* support the results that provided evidence for both endogenous and exogenous anti-amyloid activity of RP. Based on these data, we can conclude that the RP indeed inhibits pathological effects of Abeta. Together with improving memory and learning in *Drosophila*, this suggests that the RP has potential importance as a therapy for AD.

RP effects on α-synuclein expression in yeast and Drosophila models

Our next aim was to test the RP for treatment of Parkinson's disease (PD). PD is considered to be the most frequent movement disorder among neurodegenerative diseases. Typical PD symptoms include akinesia, the rigidity of facial and

skeletal muscles, resting tremor, and the loss of dopaminergic neurons (Wales et al. 2013). Surviving neurons accumulate cytoplasmic inclusions known as Lewy bodies that contain a large amount of α-synuclein in a form of fibrillar aggregates (Spillantini et al. 1997, 1998; Baba et al. 1998). Monomeric α-synuclein controls the neuronal transmission, but superexpression of this protein disrupts vesicular transport. Oligomers and protofibrils, intermediates in the aggregation process, are considered to be the most toxic species. They impair mitochondrial, lysosomal, and proteasomal function, damage biological membranes, destroy synaptic functions, and cause neurodegeneration (Outerio and Linquist 2003, Tenreiro et al. 2017). The process of α-synuclein aggregation is regarded as a major pathogenic factor and hence a promising target for potential therapeutic agents (Stefanis 2012). Alas, clinical research on dozens of proposed drugs failed to find one that demonstrated suitability for medical practice. Practical treatments remain symptomatic; no disease-exterminating ones are available (Racette and Willis 2015; Oertel and Schulz 2016; Outerio and Mestre 2019; Brás et al. 2020). Steiner et al. (2018) warn that "there is still a lack of tools to help connect pathology to dysfunction and disease state in clinical PD cases."

S. cerevisiae expressing recombinant genes of human α-synuclein is a popular system for testing for more compounds affecting α-synuclein aggregation and amyloidization. The production and aggregation of cytoplasmic vesicles in yeast cells, induced by α-synuclein, are analogous to the formation of Lewy bodies in patients with PD (Outeiro and Lindquist 2003; Soper et al. 2008; Franssens et al. 2010; Tenreiro et al. 2017). In many inherited cases, amplification of the α-synuclein locus and resulting protein overproduction causes PD. Using this phenomenon, a model was constructed to study the mechanisms of PD pathogenesis in yeast. It comprises an integrated plasmid containing two copies of the SNCA-GFP coding for a recombinant α-synuclein-GFP protein. Genes responsible for the synthesis of the hybrid protein are controlled by the galactose-driven promoter GAL1p, allowing expression of this protein to be triggered by galactose, and not by any other carbon sources, such as raffinose or glucose (Miller-Fleming et al. 2008; Tenreiro and Outeiro 2010; Tenreiro et al. 2013, 2016). This model was kindly donated to us by Dr. Outeiro.

The *Drosophila* model also holds much promise. Though the fruit flies genome does not contain endogenous α-synuclein or its orthologs (Maries et al. 2003), flies expressing human α-synuclein develop degeneration of dopaminergic neurons, formation of inclusions, such as Lewy bodies, and impaired locomotor function (Feany and Bender 2000). The resulting neurodegenerative pathology of fruit flies is similar to that observed in human PD patients.

Using the yeast model (Nevzglyadova et al. 2018), we demonstrated the effect of exogenous RP on cell viability. In galactose media, it was clearly pronounced: starting from 16 h of cell growth, RP-treatment leads to a decrease in the proportion of propidium-positive (dead) cells. α-Synuclein-mediated death in yeast follows the apoptosis pathway (Ribeiro et al. 2006) and is known to be due to mitochondrial destruction and oxidative stress (Tenreiro et al. 2017). As the RP treatment leads to better cell survival, we suggested that this effect is caused by changes in the apoptosis pathway. The accumulation of ROS is known to be a typical feature of yeast apoptosis. Applying specific dye (Deep Red), we demonstrated that α-synuclein-GFP cytotoxicity was associated with an increased proportion of ROS-positive cells during growth in galactose, while RP treatment significantly diminished this percent. In contrast, in the absence of *SNCA* expression on raffinose-containing media, the proportion of ROS-positive cells was very low and did not depend on RP presence. Western blot analysis revealed that the pellet material enriched with higher MW polymers of α-synuclein-GFP were less abundant in RP-treated cells.

To get insights into possible changes in the α-synuclein-GFP aggregates formed in RP-treated cells, we performed FRAP analysis. This method has been widely used to elucidate the structure of aggregates and the dynamics of their generation in living cells. In our experiment, FRAP assay characterizes diffusion ability of α-synuclein molecules forming the cytoplasmic inclusions, to recover fluorescence after photobleaching. Our results demonstrate that inclusions formed by α-synuclein-GFP are rather heterogeneous, which is consistent with the data obtained previously in the same model (Tenreiro et al. 2014a, b). The high recovery group (HR) has the highest rate of diffusion. This group includes relatively small aggregates, presumably the most toxic ones. After RP-treatment, the HR group considerably decreases, and the new LR (low recovery) group with almost no recovery emerges. We suggest that the RP-mediated shift in the observed groups is due to α-synuclein molecules coalescence. RP leads to depletion of small toxic HR molecules by coalescing them into large less toxic aggregates. This helps the cells to survive. Our data are consistent with the results obtained by Villar-Piqué et al. (2016), who showed that some environmental factors, such as copper, induce the formation of structurally different and less-damaging α-synuclein aggregates.

The RP-mediated drop in toxicity was also demonstrated in transgenic *Drosophila* strains after oral administration of yeast cells. We analyzed the α-synuclein level in the lysates of heads of fruit flies consuming red or white yeast. *Drosophila* fed on red yeast had smaller amounts of both soluble and insoluble forms of α-synuclein than flies consuming white yeast. Using *Drosophila* brain tissue sections, we demonstrated that RP helps to compensate for an α-synuclein-mediated reduction in the number of dopaminergic neurons of 30-day old flies. It also leads to

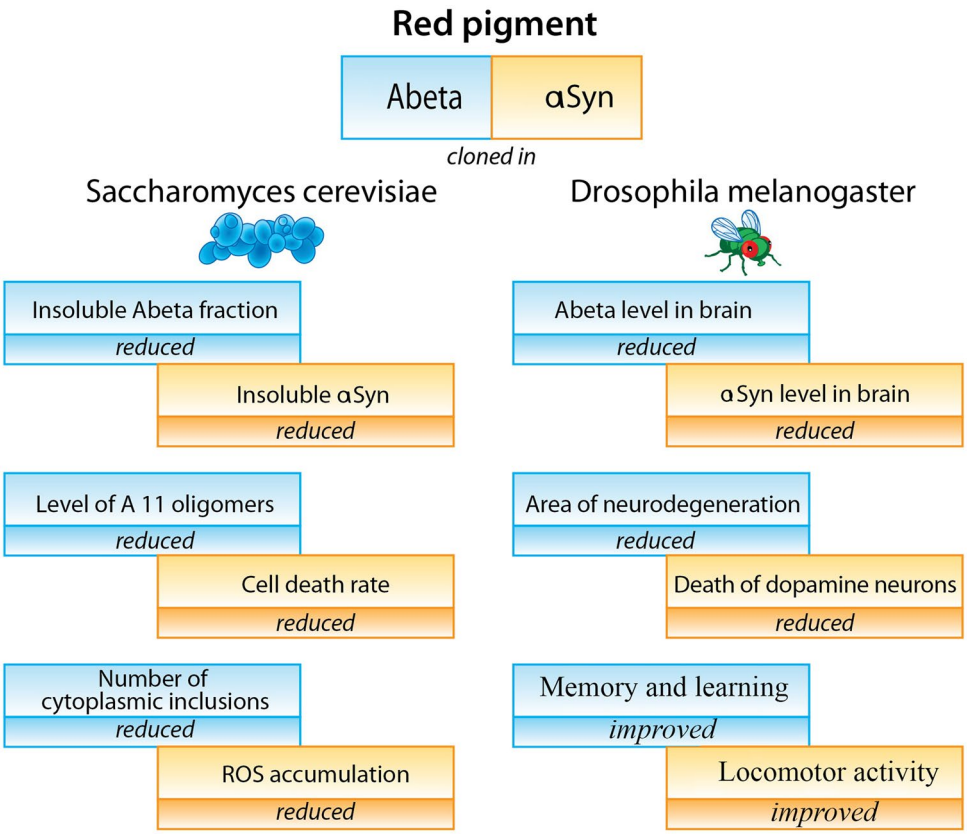

Fig. 6 RP effects on human Abeta and α-synuclein expressed in yeast and *Drosophila*

better performance in animal climbing tests estimating the locomotor ability of α-synuclein flies.

Together, these results provide evidence for RP capacity to reduce features related with pathological processes induced by Abeta and α-synuclein in both yeast and *Drosophila* models (Fig. 6).

Conclusions

1. [PSI+] strains contain numerous pellet proteins lacking in [psi-] isogenic strains. MALDI-TOF analysis permits the identification of a group of prion-dependent proteins.
2. RP diminishes amyloid amount, amyloid and prion aggregation, and amount of pellet's proteins.
3. Comparative MALDI analysis permits the identification of a set of RP-dependent proteins.
4. TEM and AFM demonstrate that RP prevents growth of amyloid fibrils in vitro.
5. MALDI-TOF analysis of synthetic RP lacking amino acids allows to clarify its structure and to propose oxidative polymerization as the way of its synthesis.
6. RP reduces pathological effects caused by human peptides involved in conformation diseases (AD and PD).

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Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

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