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Aldolase is a Target of Oxidative Modifications: Evidence from *Drosophila melanogaster*

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Abstract: **A progressive accumulation of oxidative damage to proteins has been hypothesized to be one of the factors responsible for the functional decline with age. The main objective of this study was to investigate if the age-related oxidative modifications induced by endogenously generated reactive species can alter protein function and potentially have a damaging effect on metabolic pathways during the ageing process. Accordingly, age-related changes in protein carbonylation – a marker of oxidative posttranslational modifications – were determined for cytosolic proteins from the flight muscles of *Drosophila melanogaster*. Our study establishes that the cytosolic protein, D-fructose 1,6-bisphosphate aldolase (EC 4.1.2.13) has been found to be a specific target of oxidation during ageing in the thoracic cytosol of *D. melanogaster*. A positive correlation between age of flies and the extent of carbonylation of aldolase with 9 and 22% increase at 34- and 60-days in comparison to 10-day controls was observed. In addition, a concomitant decline in enzymatic activity of aldolase was observed with 62% activity at 55-days in comparison to 10-day cohort controls, indicating that the age-associated posttranslational modifications potentially affect enzymatic activity of aldolase. Since the enzyme has a central role in carbohydrate and energy metabolism, this may potentially affect the functional efficiency of the organism.**

Index Terms**: Protein oxidation, oxidative posttranslational modifications, ageing, oxidative stress, aldolase, cytosol.**

I. INTRODUCTION

The free radical theory of ageing or the oxidative stress theory of ageing hypothesizes that the progressive accretion of oxidative damage to proteins is primarily responsible for the decline in physiological capacity of organisms during the ageing process (Harman, 1956; Krisko & Radman, 2019; Sohal & Orr, 1994; Sohal & Weindruch, 1996; Stadtman, 1992). Reactive species are produced in the body through various metabolic pathways including the electron transport chain which has been implicated as the major endogenous source of reactive species (Li et al., 2013). Since the damage to cellular macromolecules is mediated by reactive products of normal metabolism, this theory is also called the oxidative ‘‘garbage catastrophe theory’’ (Hayflick 2007).

Age-associated non-enzymatic oxidative posttranslational modifications include carbonylation, nitration, chlorination, oxidation, racemization, isomerization, deamidation, nitration, carbamylation and glycation (or glycoxidation) (Soskic et al. 2008). These modifications have been demonstrated to be amplified in various oxidative stress-mediated diseases such as diabetes mellitus, chronic renal failure, neurodegenerative diseases, atherosclerosis (Jaisson & Gillery, 2010; Krisko & Radman, 2019; Nandi et al., 2019) or autoimmune diseases (Ryan et al., 2014). These damaging effects are accelerated due to targeting of the repair process elements too as the antioxidant enzymes.

Generation of protein carbonyls – one of the dominant manifestations of oxidative protein modification – are considered to be stable marker of oxidative damage to proteins. Mechanisms that lead to the production of protein carbonyls *in vivo* are either (i) via metal catalysed oxidation or through mixed function oxidation; (ii) by adductation with lipid peroxidation products; (iii) reactions with sugars (glycation) or their oxidation products (glycoxidation) with the glycine residues (Estebauer et al., 1991; Stadtman, 1990; Stadtman, 1992). Studies have shown that *in vitro* induced carbonylation (induced by oxidative stress

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conditions) can lead to increased protein aggregation (Tanase et al., 2016).

The mitochondria in postmitotic cells use O_2 at a high rate in the electron transport chain releasing oxygen radicals due to electron leakage (with up to 3% electron leakage with age) (Li et al., 2013) which may surpass the abilities of the defence mechanism including the cellular antioxidants (Miquel & Fleming, 1986). Also, since it has been established that the complex I-dependent superoxide is entirely released into the mitochondrial matrix and that no demonstrable quantites leak from intact mitochondria (Muller et al., 2004), many studies have focused on the mitochondria to study the implications and consequence of the oxidative onslaught. In flies, since flight is the main manifestation of physical activity, the flight muscle is the most apparent site for oxidative modifications during ageing. A few studies directed towards the proteins of the flight muscle mitochondria of flies showed that aconitase and adenine nucleotide translocase 1 are major targets of oxidation during ageing and hyperoxia in the *Musca domestica* and *Drosophila melanogaster* (Das et al., 2001; Yan et al., 1997; Yan & Sohal, 1998). Both proteins were reported to undergo increase in carbonylation with concomitant decrease in their respective enzymatic activities as a function of age as well as under hyperoxia. However, it has not yet been established in flies whether proteins in the non-mitochondrial fractions of the cell including the cytosol are also targeted during the process of ageing and, if so, what is the pattern and extent of such damage.

The purpose of the present study was, thus, to determine (i) whether any age-associated oxidative posttranslational modifications occurs among the cytosolic proteins of the flight muscles of *D. melanogaster*; (ii) whether the oxidative damage is selective or random, in that all or many proteins are targeted, or only a few are affected; (iii) if such damage is homologous to age- and oxidative stress-mediated damage in other insects and mammals as reported by other groups, and (iv) if such posttranslational modifications to proteins leads to a concomitant decrease in their enzymatic activity. This study was conducted on *D. melanogaster* flies of *y w* (yellow body, white eyes) strain to complement and extend previous investigations (Das et al., 2001) which focused on only the mitochondria of this strain. Cytosolic proteins from the flight muscles of *D. melanogaster* were analyzed using an immunochemical probe for oxidatively modified (carbonylated) proteins to investigate the role of protein carbonylation in ageing of flies.

II. MATERIALS AND METHODS

A. Materials

BSA Fraction V, Acrylamide/bisacrylamide (40%), *N,N,N',N'*-tetramethylethylenediamine (TEMED), 2,4-dinitrophenyl hydrazine (DNPH), Coomassie brilliant blue R250, ammonium persulphate, ethylenediaminetetraacetic acid (EDTA) tetrasodium salt, sodium dodecyl sulphate (SDS), glycine, polyclonal rabbit anti-dinitrophenyl (DNP) and horseradish peroxidase-conjugated goat anti-rabbit antibodies were obtained from Sigma Chemical Co. Ampholyte (pH 3-10) and protein standards were from Bio-Rad. Bicinchoninic acid (BCA) protein assay reagent was from Pierce Laboratories. Methanol, ethanol, potassium chloride, polyoxyethylene-20-sorbitan monolaurate (Tween 20) and ethyl acetate were from Sisco Research Laboratories, India. PVDF (Immobilon™-P) membrane was purchased from Millipore. ECL™-Plus was purchased from Amersham Pharmacia Biotech.

B. Maintenance of Drosophila flies

All experiments were conducted on male *D. melanogaster* flies of *y w* (yellow body, white eyes) strain. Adult male flies were segregated immediately after emergence from pupae and maintained in groups of 25 at 50% relative humidity conditions in standard plastic vials at 25°C. For the first 25 days, fresh food consisting of 0.7% (v/v) propionic acid/phosphoric acid (10:1) and methyl paraben as mold inhibitor added to a cornmeal/agar/yeast medium were given every other day; subsequently, the food was changed each day (Mockett et al., 2002).

C. Isolation of flight muscle cytosol

All procedures were conducted at 4°C unless otherwise stated. Thoraces of male *D. melanogaster* y w flies were pounded in a chilled mortar pestle in buffer containing 0.16 mM $KHCO_3$, 154 mM KCl, pH 7.0, and 1 mM EGTA. The cytoskeleton was removed by filtration through SpectraMesh® and the filtrate centrifuged at 4°C for 3 min at 300 g to pellet the cellular debris. The soup was gently drawn off and the mitochondrial pellet separated by centrifugation at 3000 g (Van Den Bergh, 1967). The post-mitochondrial soup was centrifuged at 4°C for 30 min at 25,000 g to pellet the organelles. The supernatant containing the cytosolic fraction was used for all studies.

D. Dinitrophenylhydrazine (DNPH) treatment of the cytosolic proteins

Cytosolic proteins were derivatized with DNPH (0.2M) for 1 h, pelleted with 10% (w/v) ice-cold trichloroacetic acid and washed with a solvent mixture of ethanol and ethyl acetate (1:1, v/v) (Levine et al., 1990). The proteins were dissolved in 20 mM Tris-HCl buffer, pH 6.8, containing 0.2% (w/v) sodium dodecyl sulfate (SDS). A cytosolic protein sample treated with 2M HCl was used as negative control.

E. Cytosolic protein analyses by immuno-blotting

The proteins were separated in 10% SDS-PAGE under reducing conditions (Laemmli, 1970) and the resolved proteins were either stained with Coomassie brilliant blue R250 or proceeded to immuno-detection. Proteins were transferred to PVDF (Immunobilon™-P) membrane by the wet transfer method (Towbin et al., 1979).

Immunochemical detection of carbonylated proteins was performed (Keller et al. 1993; Shacter et al. 1994) by treating the membrane with 5% (w/v) non-fat milk and Tween 20 (TBST) in Tris-buffered saline. After washing thrice with TBST, the membrane was incubated with the primary antibody. The membrane was then exposed to secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG in TBST buffer containing 0.2% BSA). The ECL™ Plus western blotting detection kit was used to detect the carbonylated proteins. The amount of carbonylation was calculated by densitometric scanning of the immuno-stained bands along with standards of oxidized BSA treated similarly with DNPH and processed for western blotting.

F. Purification and identification of the carbonylated proteins

The DNPH treated cytosolic proteins were prepared for IEF by incubating the proteins for 30 min in buffer with 3% ampholyte, pH 3-10, 4 M urea, 1% β-mercaptoethanol and 2% (v/v) Nonidet P40. Proteins were loaded on an IEF gel and run according to Bollag et al. (1996). For the 2D-PAGE run, the gel was incubated for 30 min in 62.5 mM Tris-HCl buffer, pH 6.8, containing 5% β-mercaptoethanol and 2.3% (w/v) SDS and the gel strips were run on a 4-20% gradient gel along with one lane containing DNPH treated cytosolic proteins as positive controls. The resolved proteins were transferred to PVDF membrane and proteins of interest identified by western blotting using anti-DNP antibodies. The proteins of interest were identified by mass to charge ratio of the trypsinized fragments at the University of Southern California core sequencing facility and subsequent database search.

G. Measurement of aldolase activity

Aldolase was assayed using the continuous indirect spectrophotometric rate determination method of Bergmeyer (1976) in a Shimadzu UV 1800 spectrophotometer. Briefly, aldolase activity for fructose 1,6-biphosphate was determined by a coupled assay. A 100 mM Tris-HCl buffer, pH 7.4 was used to prepare all the reagents.

H. Measurement of protein content

The protein content was measured using Pierce bicinchoninic acid protein assay kit and BSA as a standard (Smith et al., 1985).

I. Statistical Analysis

Data presented are mean ±SD of 2 to 3 independent experiments. Measurement of carbonylated proteins and enzyme activities were compared statistically using one-way analysis of variance (ANOVA) and post hoc analysis. *P* values of <0.05 were considered as significant and values of <0.0001 were considered as highly significant.

III. RESULTS

A. Pattern of carbonylation of cytosolic proteins

To detect specific protein(s) demonstrating age-associated protein oxidative posttranslational modifications, the cytosolic fraction from male *D. melanogaster* thorax, ranging from 9 to 63 days of age ((9-11)-, (28-37)- and (55-63)-day-old flies for young, middle and old age groups, respectively), were resolved by SDS-PAGE following DNPH derivatization. Staining with Coomassie blue was used to visualize the total protein profile while immunoblotting was used to detect the carbonylated proteins. Immuno-blotting indicated that two protein bands of subunit molecular masses 40±2 and 85±5 kDa were differentially carbonylated with age of the flies (Fig. 1, lanes 4, 5 and 6). However, many other protein bands which were even more prominently stained with Coomassie blue did not show any immuno-reactivity to anti-DNP antibodies indicating the specificity and selectivity of the posttranslational modifications with age. The negative control comprising of cytosolic proteins loaded onto SDS-PAGE without DNPH treatment did not show any immuno-stained band (Fig. 1, lane 3), confirming the specificity of the antibody.

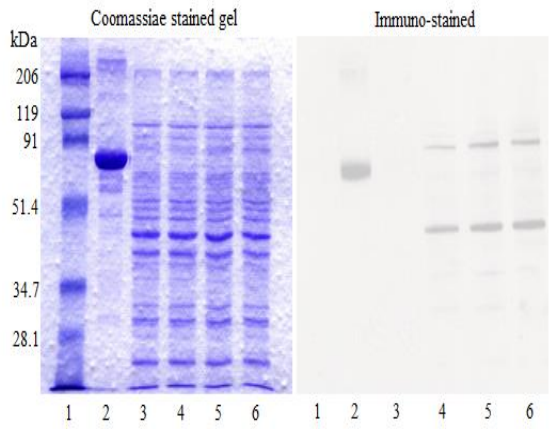

Fig. 1. Western blotting of carbonylated proteins in the cytosol from flight muscles of *D. melanogaster*. Lane 1: standard protein markers, Lane 2: DNPH treated oxidized BSA (positive control), Lane 3: negative control, Lanes 4, 5 and 6 are 11-, 34- and 60-day-old cytosol samples from thoraces of *D. melanogaster*.

B. Quantification of carbonylation levels in susceptible proteins

The effect of age on the extent of carbonylation of the 40 kDa protein was estimated by densitometric scanning of the X-ray films used for the chemiluminescence assay (Fig. 2). Study of the band density showed a positive correlation between age of flies and the extent of carbonylation. The amount of carbonylation of the 40 kDa protein increased by 9 and 22% at 34 and 60 days of age of the flies, respectively, with respect to 11-day control (Fig. 2, Panel A). The corresponding values for the 85 kDa protein were an increase by 21 and 58%, respectively.

In a separate study, the concentration of carbonyl groups in the 40 kDa protein was estimated with oxidized BSA as standard. The carbonyl content of this protein was estimated densitometrically to be approximately 146.7, 160.2 and 179.6 mmol/mol of protein in 11-, 34- and 60-day-old flies, respectively (Fig. 2, Panel B). The increase in carbonylation of the 40 kDa protein with age was statistically highly significant (P<0.0001) following one-way ANOVA and post hoc tukey's t test (values are the mean of quadruple measurements of two independent experiments).

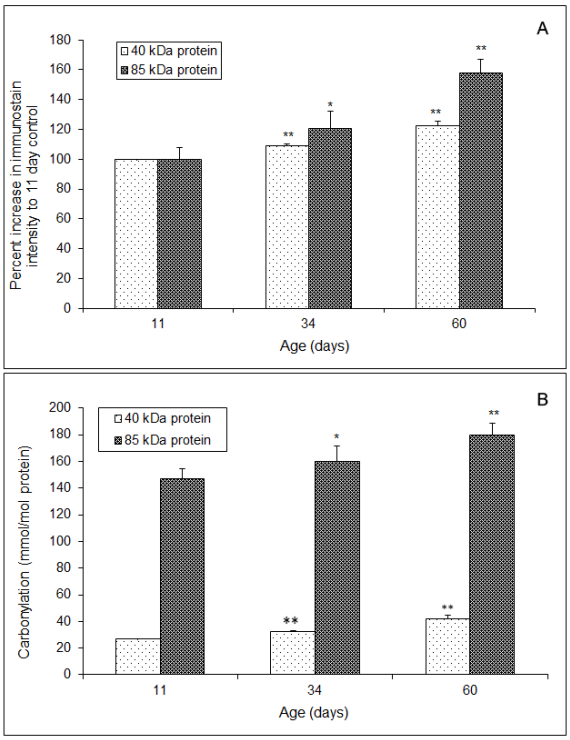

Fig. 2. Quantitation of age-associated increase in carbonylation of oxidatively modified cytosolic proteins. Panel A: The percent increase in carbonylation with respect to young flies (11-days-old) was calculated from densitometry scanning of the immuno-stained bands. **Panel B**: Quantification of the concentration of carbonyl groups in the targeted proteins.

C. Purification and identification of the oxidatively modified proteins

2-D PAGE and gel tryptic digestion followed by mass spectrometry analysis at the University of Southern California core sequencing facility was used to identify the two selectively carbonylated proteins. The 40 kDa protein was identified as fructose-1,6-bisphosphate aldolase γ (EC 4.1.2.13), an enzyme involved in carbohydrate metabolism. The 85 kDa protein showed some internal sequences repeating several times with a couple of unique regions. However, these sequences did not match with the database and the 85 kDa protein could not be identified.

D. Effect of chronological age of the flies on aldolase activity

To estimate the role of age-associated increase in carbonylation of aldolase on its functional ability, the enzymatic activity was quantified in the cytosol of the fly thoraces. The results of at least triplicate measurements of two independent experiments showed highly significant decrease (P<0.0001) in enzymatic activity with age of the flies (Fig. 3). In comparison to the 10-day-old flies, activity decreased to 91.8, 84.9, 79.7 and 62.3% in the 16-, 30-, 40- and 55-day-old flies.

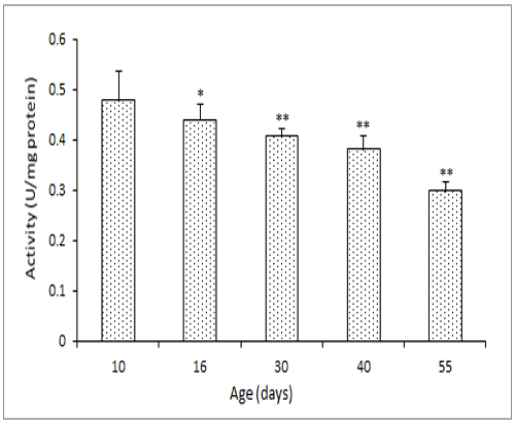

Fig. 3. Quantitation of aldolase activity with age in the thoraces of *D. melanogaster*. Aldolase activity was assayed spectrophotometrically using *D. melanogaster* thoraces of different ages by estimating the rate of decrease in absorbance of NADH at 340 nm in the presence of the co-enzymes, glycerol-3-phosphate dehydrogenase and triose phosphate isomerase. Values represented are the mean±SD of at least triplicate measurements of two to three independent experiments. Pairwise mean comparison were performed by post-hoc Bonferroni's multiple comparison test, following one-way ANOVA.

IV. DISCUSSION

The present study attempts to evaluate the age-associated posttranslational modification of the cytosolic proteins in the

flight muscle of *D. melanogaster*. To extend the findings of an earlier study (Das et al., 2001) where the enzyme, aconitase, was found to be selectively carbonylated in the thoracic mitochondria of *D. melanogaster* y w strain, the cytosol was investigated in the same strain to determine if the reactive species produced in the cell during cellular metabolism also target proteins in other sites/organelles of the cells besides the mitochondria.

D-fructose 1,6-bisphosphate aldolase γ (EC 4.1.2.13) has been shown to be selectively targeted in the fly flight muscle cytosol by carbonylation which increased progressively with the age of the flies. Many other proteins which were also abundant in flight muscle as demonstrated by Coomassie blue staining did not exhibit perceptible carbonylation with age of the flies indicating selectivity of protein posttranslational modifications. Preliminary hyperoxic experiments also showed these two proteins bands to be selectively carbonylated (results not shown) with an additional ~100 kDa protein exhibiting perceptible carbonylation at 3 days of hyperoxia. To gain an understanding of the impact of the age-associated increase in carbonylation of aldolase on its enzymatic activity, the activity was measured. Concomitant decreased enzymatic activity of aldolase with age was observed in *D. melanogaster*. The 85 kDa protein band, showing repeat sequences is most probably an artifact of *in vitro* treatments as DNPH or SDS-PAGE treatments. Interestingly, an approximately 85 kDa protein has also been observed by other groups (Zhang et al., 1995) during the course of purification of recombinant aldolase isozymes expressed in *E. coli* as can be observed in their published Fig. 1, Lane 3 data. This group reasoned that the 85 kDa protein is an artifact of the purification protocol, presence of this protein in our study also indicates that the 85 kDa protein (with internal repeats) is probably a multimeric artifact and such polymerization may be an inherent feature of this protein. Further studies in this aspect is needed to gain a better insight.

That aldolase in the cytosol of *D. melanogaster* loses its activity along with concomitant extensive carbonylation invites extensive deliberation. The active-site and/or substrate-binding sites of aldolase have amino acid residues as lysine-107, -146, -229, glutamic acid-187 and arginine-55, -148 (Horecker et al., 1963; Melek et al., 1985; 1988) all of which are known to be susceptible to oxidative modifications. The C-terminal also has a conserved tyrosine residue which is essential for its substrate binding. Presumably posttranslational modifications as carbonylation are targeted at one or more of these amino acids. It is also known that *Drosophila* aldolase, unlike rabbit muscle aldolase, has the active site cleft covered by a fold of polypeptide chain formed by residues 345-363 (Hester et al., 1991). This peptide arm might have formed a physical barrier inhibiting access to the active site by reactive oxygen species but since the *Drosophila* enzymatic activity is observed to decrease with age, it may not be the case. Presumably this may be due to need of the substrates to access the active site. However, it is possible that the level of oxidative damage is more in rabbit aldolase than in the *Drosophila* aldolase. Further comparative studies would elucidate the point.

There is plethora of work on reactive species-mediated and/or age-associated damage to cellular macromolecules. A number of such reports establish that some proteins are selectively modified. Such findings have been reported from diverse species like yeast, insects (*Drosophila* and *Musca*) and mammals (as rats, mice and rhesus monkeys) (Cabiscol et al., 2000; Das et al., 2001; Hussain et al., 2006; Jana et al., 2002; Kanski et al., 2003; Kanski et al., 2005a; 2005b; Poon et al.. 2006; 2007; Reverter-Branchet et al., 2004; Yan et al., 1997; Yan & Sohal 1998).

The phenomenon of aldolase being targeted by reactive species during ageing is observed in diverse species. Studies on the ageing rat diaphragm showed HNE-mediated modification of aldolase, amongst other proteins (Hussain et al., 2006). In a proteomic study on rat skeletal muscle and cardiac tissues (both whole heart homogenate as well as heart mitochondria), a few proteins showed nitration with age including alpha-fructose aldolase (Kanski et al., 2003; 2005a; 2005b). The findings led the authors to suggest that nitration of key proteins could, in part, be responsible for the age-associated decline in functioning of the muscle and cardiac tissues. Aldolase has been reported to be carbonylated with increasing age of *Saccharomyces cerevisiae* (Reverter-Branchet et al., 2004) and in the mice brain (Poon et al., 2005; 2007). Formation of 3-nitrotyrosine residues in aldolase in ageing rat cerebellum (Gokulrangan et al., 2007) and carbonylation and nitrotyrosine residues of mice olfactory bulb aldolase (Vaishnav et al., 2007) and nitration as a result of nitric oxide-induced inflammation in the liver (Aulak et al., 2001) has been reported. However, activity studies of the posttranslational modified proteins compromising of *inter alia* aldolase were not carried out by any of these groups although speculations on the role of oxidative posttranslational modifications in potentially triggering the age-dependent apparent attrition has been made. This is especially true of proteomic studies. In view of results from rat heart and skeletal muscle where it has been established that not all proteins lose its activity in spite of being targeted by reactive species and/or oxidative stress (Yarian et al., 2005), such speculation about contribution of oxidative modifications towards cellular dysfunction by these authors may need to be validated. Since the concomitant aldolase activity was not measured in almost all of these reports, it remains difficult to speculate from the present state of the art if oxidative posttranslational modification to aldolase always results in affecting its functional activity. In only one study was activity assay performed where it was observed that lipopolysaccharide increased carbonylation of aldolase in rat diaphragm along with concomitant decrease in activity of aldolase (Barreiro et al., 2005). However, since the stimulus was an induced one which would depend on the level of

lipopolysaccharide administration, it may not mimic physiological levels of oxidative stress observed under normal ageing. In view of such arguments, the possibility of aldolase as a marker of age and/or oxidative stress cannot be speculated upon with any degree of certainty with the present status where the data is still too sketchy. Therefore, further investigation to forward the cited studies are warranted.

Aldolase has a central position in carbohydrate metabolism, participating in anabolic (Calvin cycle and gluconeogenesis) as well as catabolic (glycolysis) pathways. It is responsible for the split of the six carbon fructose diphosphate into two three-carbon compounds, glyceraldehyde-3-phosphate, an aldehyde and dihydroxyacetonephosphate, a ketone in the glycolytic pathway. It may be presumed that such age-associated posttranslational modifications to aldolase will impair the crucial cellular processes of ATP generation and energy dependent processes.

CONCLUSION

In this study, aldolase in *D. melanogaster* has been demonstrated to be selectively oxidatively modified during ageing. There was also a concomitant age-associated decrease in its activity. This work supports the growing body of evidence on selectivity of protein oxidation. However, further work needs to be performed to establish if this phenomenon is specific to *D. melanogaster* only or is found in other insects and mammalian species also. Our findings indicate that the age- and oxidative stress-associated posttranslational modifications do not seem to be confined to the mitochondria only but may be a more spread out and diverse phenomenon.

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