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Anti-aggregation effect of Ascorbic Acid and Quercetin on aggregated Bovine Serum Albumin Induced by Dithiothreitol: Comparison of Turbidity and Soluble Protein Fraction Methods

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Abstract

Studies on the anti-aggregation of dithiothreitol (DTT) induced - protein is generally determined by the fraction soluble (non-aggregated) protein. While the turbidity method is commonly used in studies of anti-aggregation, in which protein is induced by heat, in this study, both methods are compared in observing the anti-aggregation activity of ascorbic acid and quercetin toward bovine serum albumin induced by DTT. The DTT is a reducing agent for protein disulfide bonds and capable of inducing protein aggregation at physiological pH and temperature. The work was performed by the formation of Bovine Serum Albumin (BSA) aggregates induced by DTT under physiological conditions, which are pH 7.4 and 37°C. The aggregated protein profile was observed using the turbidity method at the end of incubation and measuring the difference of concentration between the fraction of soluble protein before and after incubation. The measurement was carried out using a spectrophotometer UV-Vis. The results indicate that both methods show similar inhibition profiles. The potential inhibition of ascorbic acid (AA) toward BSA protein aggregation induced by DTT increased along with incubation time. While quercetin shows the highest inhibition at 12 hours but decreased at 18 hours, this study reveals that both methods can observe the anti-aggregation activity of ascorbic acid and quercetin.

1. Introduction

The stability of the natural structure of a protein is strongly influenced by its disulfide bonds [1, 2, 3, 4]. The reduction of disulfide bonds significantly decreases the stability of protein structures [5, 6, 7]. Since the stability of proteins is substantially increased by naturally occurring disulfide cross-links, the cleavage of disulfide bonds will trigger instability of protein structure, which further triggers aggregation. While in many studies, dithiothreitol (DTT) is reported to be a thiol-based protein disulfide reducing agent, which is well-known for investigating the cleavage of disulfide bonds [8, 9, 10]. Furthermore, Yang et al. [11] showed that DTT could induce aggregation of BSA and lysozyme proteins. BSA has 17 disulfide bonds and one free cysteine [12]. The structures of BSA and DTT are presented in Figures 1 and 2, respectively.

Turbidity measurement is commonly used in monitoring protein aggregation [13]. That is because the increase in turbidity shows that the protein has undergone aggregation [14]. Pulford *et al.* [15] measured the turbidity of the aggregations of heat-induced *bovine casein* and *crystallin* proteins at an excitation wavelength of 360 nm. However, for anti-aggregation studies using bioactive compounds, measurements were carried out at higher wavelength to avoid interference from chromophore absorbance, as suggested by Baier *et al.* [16] to conduct turbidity measurement at an excitation wavelength of 600 nm. Also, Yang *et al.* [11] observed the activity of DTT-induced protein aggregation by measuring the concentrations of the fraction of soluble protein before and after the aggregation processes (non-aggregated) and observing the difference between them. Borzova *et al.* [17] reported that the anti-aggregation

activity of *Chaperones* on BSA protein could also be observed by looking at the non-aggregate protein.

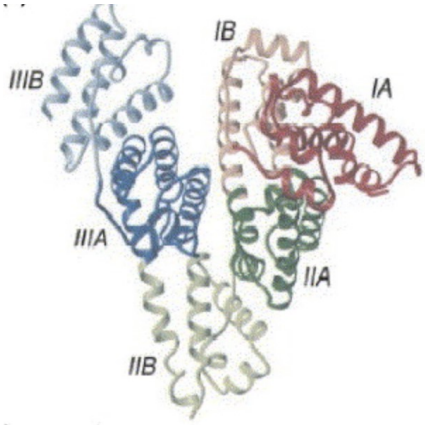

Figure 1. Structure of bovine serum albumin. The heart-shaped serum albumin molecule consists of three homologous α-helical domains (I, II, III). Each domain contains two subdomains (A and B) that share common structural motifs [18]

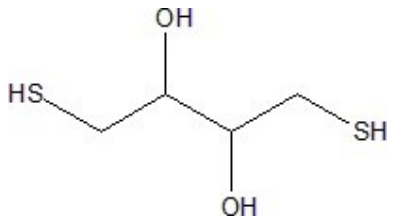

Figure 2. Structure of dithiothreitol (DTT)

There are many reports on the potential bioactive compounds as anti-aggregation agents. Research conducted by Alam *et al.* [19] reported that ascorbic acid inhibited *aggregation of human insuli*n, observed by ThT and ANS fluorescence methods. In addition, Wang *et al.* [20] also showed that quercetin was able to inhibit *bovine insulin* protein aggregation observed using the ThT fluorescence method, and the aggregate structure formed was identified using a transmission electron microscope (TEM). The structure of ascorbic acid and quercetin can be seen in Figure 3.

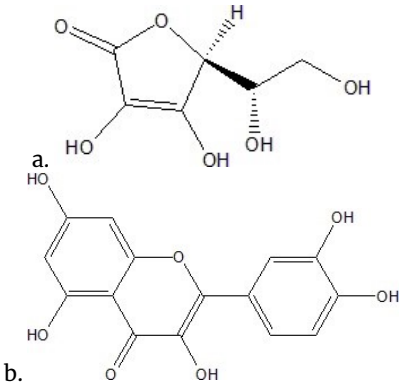

Figure 3. Structures of (a) ascorbic acid and (b) quercetin

Based on the elaboration, it is necessary to test the ability of both methods to monitor protein aggregation. The methods are turbidity at 600 nm and soluble protein fraction (non-aggregated) at 280 nm on observing the aggregation of BSA protein induced by DTT in the presence of ascorbic acid and quercetin as inhibitors.

Turbidity is defined as the attenuation of the incident beam by light scattering and thus can be estimated either straightforwardly, via measurement of the loss of intensity of transmitted light or secondarily, by the integration of the angle-dependent scattering at a fixed distance. The relatively straightforward nature of the transmission measurement is that requiring only a spectrophotometer or plate reader. The simplification of light scattering (due to the lack of a requirement for an extrinsic label) has made transmission-based turbidimetric assays the default 'basic' standard for recording protein aggregation kinetics [14].

At wavelength 280 nm, the aromatic amino acids tryptophan (Trp) and tyrosine (Tyr) exhibit strong light absorption, and to a lesser extent cysteine groups forming disulfide bonds (Cys–Cys) also absorb. Consequently, the absorption of proteins and peptides at 280 nm is proportional to the content of these amino acids. Apart from their intrinsic absorptivity, proteins will absorb UV light in proportion to their concentrations. This relationship has been exploited for the spectrophotometric determination of protein concentrations, and it is defined by the Beer-Lambert law (or Beer's law). Beer's law describes the dependence of a protein's absorbance on its absorptivity coefficient, its concentration, and the pathlength of the incident light:

$$A = \varepsilon cL$$

where:
A: absorbance of the sample (unitless)
ε: molar extinction coefficient or molar absorptivity of the protein ($M^{-1} cm^{-1}$)
c: concentration of the protein (molar units, M)
L: light pathlength (cm)

The ease of the turbidity method on anti-aggregation studies will be very beneficial in exploring the capacity of candidate compounds for further anti-aggregation studies.

2. Material and Methods

All the materials were bought from Sigma Aldrich: bovine serum albumin (BSA), ascorbic acid, quercetin, dithiothreitol (DTT), and phosphate buffer saline.

2.1. Aggregation process of BSA protein induced by DTT

The formation of aggregates follows the method of Yang *et al.* [11]. Each mixture contained 40 µM BSA protein in 20 mM phosphate buffer (pH of 7.4) and 10 mM DTT. The step was adding ascorbic acid (AA) and then incubated at 37°C for 20 hours. The same procedure was applied for the bioactive compound quercetin (Q) by replacing ascorbic acid with quercetin.

2.2. Measurements of turbidity and soluble protein fraction

The aggregated BSA protein mixtures with and without the presence of an inhibitor were measured their turbidity using the UV-Vis spectrophotometer at a

wavelength of 600 nm [16]. Then, the centrifugation of mixtures was carried out in order to separate aggregate and soluble protein fraction. The fraction of soluble protein in the supernatant was determined using the UV-Vis spectrophotometer at a wavelength of 280 nm [11].

2.3. Aggregate analysis

The aggregated protein profile can be seen from the mixture turbidity and the difference between the fraction of soluble protein before and after incubations.

$$\%\ inhibition = \frac{Aggregate_{control} - Aggregate_{sample}}{Aggregate_{control}} \times 100\%$$

Notes:
Aggregate $_{control}$ = Difference of BSA+DTT absorbance before and after incubations
Aggregate $_{sample}$ = Difference of BSA+bioactive+DTT absorbance before and after incubations

3. Results and Discussion

3.1. Scanning absorbance of DTT-induced aggregated BSA

The BSA protein aggregation was carried out according to Yang *et al.* [11], performed close to physiological pH and temperature. Measurements were undertaken at various incubation times to see the effect of DTT on inducing BSA protein aggregation as incubation time increased. Figure 4 shows the increase in absorbance along with increasing incubation time.

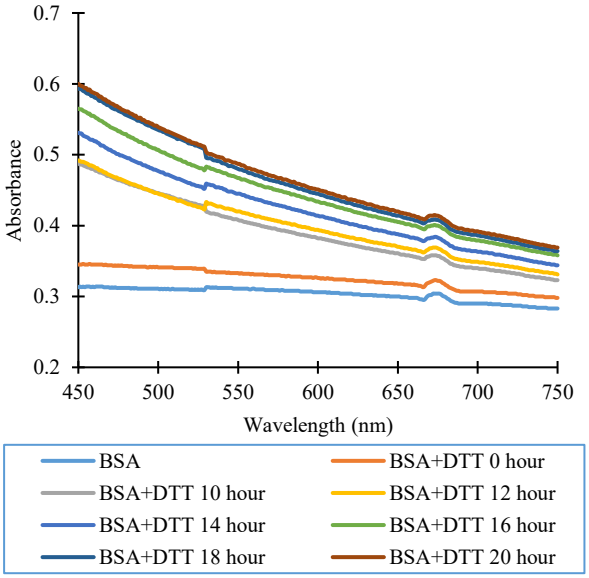

Figure 4. UV-VIS spectra of the aggregated BSA protein for various incubation times at wavelength range between 450 and 750 nm

Figure 4 shows the BSA aggregation process induced by DTT during incubation times. The increasing absorption quantity indicates high turbidity [14]. At a wavelength of 450 nm, the absorbance is significantly high, but at this wavelength, it is concerned that there is an interference of the compound absorption observed as an inhibitor. The turbidity measurement was chosen at a wavelength of 600 nm as suggested by Baier *et al.* [16], because measurement at higher wavelength avoided chromophores absorption, so it can be concluded that the increasing absorbance at a wavelength of 600 nm is an effect of the increasing amount of aggregates formed.

3.2. Turbidity Profiles of Aggregated BSA With and Without Inhibitors

The turbidity measurements using the UV-VIS spectrophotometer of each aggregated mixture were measured at 600nm, both with and without the presence of ascorbic acid and quercetin as inhibitors. Figure 5 shows the turbidity profile of each aggregated mixture.

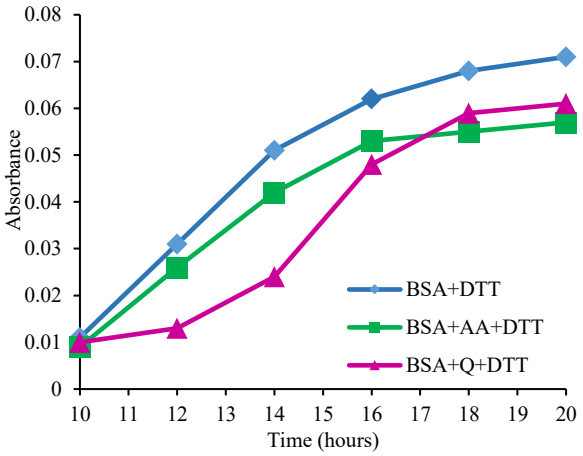

Figure 5. Turbidity profiles of aggregated mixtures observed every 2 hours started from10 hours of incubation and measured at 600 nm wavelength.

In Figure 5, it is shown that the turbidity increases for all samples throughout times. Sample with quercetin shows the lowest turbidity at the beginning but increases significantly after 16 hours. On the contrary, the inhibition profile of ascorbic acid is not strong at the beginning, but the activity increases by times. These data indicate that the inhibition mechanisms of quercetin and ascorbic acid to prevent protein aggregation are different. The higher number of phenolic groups of quercetin probably plays the primary role of this activity. The quercetin mechanism, as anti-aggregation, was reported through the prevention of conformational changes [21].

Further, it is said that the mechanism proposed is direct interaction with the misfolded proteins causing the stabilization of oligomeric species and inhibition of fibril growth. Zhu et al. [22] added that quercetin also could make covalently binding α-syn, increasing its hydrophilicity and, therefore, inhibiting the aggregation. The mechanism of ascorbic acid as the anti-aggregation agent was proposed by Patel et al. [23]. The study used spectroscopic and computational characterizations to observe the behavior of ascorbic acid-binding to hen egg-white lysozyme (HEWL). It was proposed that ascorbic acid bound to the aggregation-prone beta domain of HEWL, stabilized the partially unfolded conformation and prevented further conformational changes leading to fibrillation.

3.3. Comparison of two methods for the inhibitory activity of Ascorbic Acid and Quercetin as an anti-aggregation protein.

The measurement of inhibition percentage was performed using two methods. They were turbidity and soluble protein fraction methods. Turbidity measurement was conducted by directly measuring the aggregated mixture at 600 nm by the spectrophotometer. Measurement of soluble protein fraction (non-aggregated) was performed at a wavelength of 280 nm after the aggregates were formed and separated by centrifugation to get the soluble fraction. The percentage of inhibition was measured after 12 and 18 hours of incubation time based on a different profile of the turbidity in the previous study (Figure 5). Since the turbidity method showed the unique profile, it is necessary to see the inhibitory profile of ascorbic acid and quercetin after 12- and 18-hours incubation using a soluble protein fraction method.

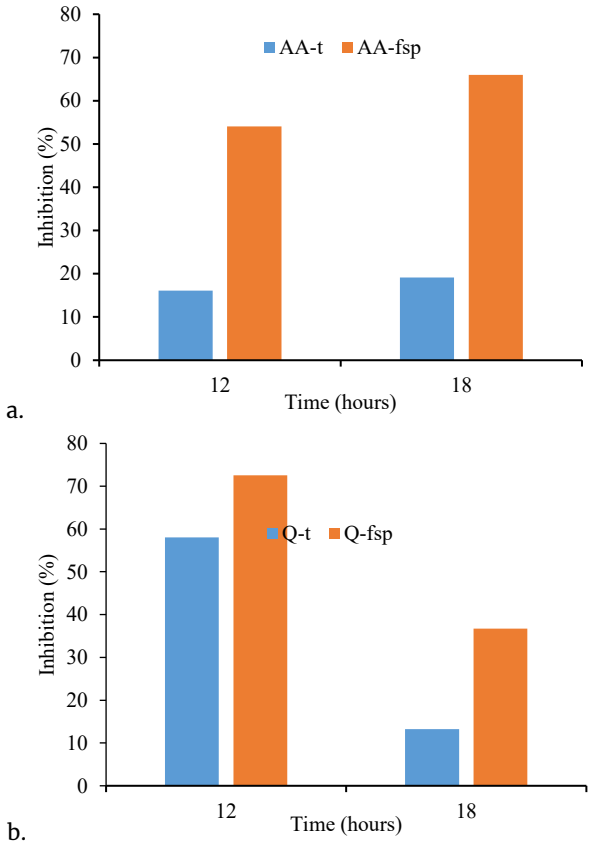

Figure 6. The comparison of percent inhibition aggregation of DTT-induced BSA protein by ascorbic acid (A) and quercetin (B), measured using turbidity (t) and a fraction of soluble protein (fsp) methods

Figure 6A shows that the inhibition percentage of BSA protein aggregation by ascorbic acid increased along with time by both turbidity and fraction of soluble protein methods. While Figure 6B presents the potential inhibition of protein aggregation by quercetin is significantly high at 12 hours of incubation and then decreases after 18 hours. Even though the value of inhibition percentage by soluble protein fraction method looks higher than that of turbidity, the profiles of both methods are similar. Interestingly, both methods can portray the unique behavior of quercetin, shown by the significant profile at 12- and 18-hours incubation times.

The reason why quercetin is more reliable at the 12 hours and decreases with prolonged incubation time, probably relates to the solubility of quercetin in the buffer. In this study, phosphate buffer saline (PBS) was used as a solvent. Quercetin is slightly soluble in aqueous buffer. For maximum solubility in aqueous buffer, quercetin must be dissolved first in dimethyl sulfoxide (DMSO) and then diluted with selected aqueous buffer. Quercetin has a solubility of about 1 mg/ml in a DMSO: PBS at 1: 4 [24]. However, dissolving quercetin is not recommended for more than a day, as stated in Cayman's chemical product information. The lower solubility of quercetin in PBS might contribute to the stability of this compound that can hold its activity in a more extended period.

On the contrary, ascorbic acid is a type of compound that dissolves well in water. Ascorbic acid also dissolves in phosphate buffer [25]. Even if ascorbic acid was hydrolyzed in a prolonged time, the derived compound of ascorbic acid was reported to have anti-aggregation activity [26]. The ascorbic acid derivative products in the form of dehydroascorbic and dicogluconic acids inhibit the formation of amyloid fibrils in insulin. Further studies are needed to ascertain what derivative products are produced from bioactive used (ascorbic acid and quercetin) at prolonged incubation.

Even though both methods portray similar profiles, there is a difference in inhibition percentage rates between turbidity and soluble protein fraction methods. The difference amount of percent of inhibition result comes from the difference in calculation approach. In the turbidity method, the total aggregate is obtained from partial protein affected by DTT in a specific time, while the soluble protein fraction method involves the whole protein contained in the solution. Therefore, the number of percent of inhibition looks higher at the soluble fraction method.

In this study, the maximum inhibitory capacity of ascorbic acid and quercetin on inhibiting aggregation of DTT-induced BSA protein was not carried out. Therefore, the comparison of inhibitory capacity between ascorbic acid and quercetin cannot be fully understood. Further studies are needed in order to gain a full understanding on the differences of the inhibitory capacity between ascorbic acid and quercetin on inhibiting aggregation of DTT-induced BSA protein, especially concerning the mechanism of protein aggregation inhibition by quercetin displaying very high activity at early incubation but then decreasing significantly at a longer time (showed by data at 18 hours). After all, these data show that both methods are equally capable of observing the anti-aggregation activity of quercetin and ascorbic acid toward BSA induced by DTT.

4. Conclusion

This study shows that both methods, turbidity and soluble protein fraction, provide similar information to portray the activity of an anti-aggregation protein,

especially on BSA protein induced by DTT. This study also shows a similar profile of quercetin and ascorbic acid on their mechanisms to inhibit the aggregation of BSA protein. The activity of ascorbic acid on inhibition of the protein aggregation induced by DTT increases at a more extended incubation period. However, the unique profile is showed by quercetin, which is quite high at early incubation, then decreases at prolonged incubation time.

Reference

[1] Thomas E. Creighton, Disulfide bond formation in proteins, in: *Methods in Enzymology*, Academic Press, 1984, pp. 305-329 https://doi.org/10.1016/0076-6879(84)07021-X

[2] Philip J. Hogg, Disulfide bonds as switches for protein function, *Trends in Biochemical Sciences*, 28, 4, (2003), 210-214 https://doi.org/10.1016/S0968-0004(03)00057-4

[3] Nandini Sarkar, Manjeet Kumar and Vikash Kumar Dubey, Effect of sodium tetrathionate on amyloid fibril: Insight into the role of disulfide bond in amyloid progression, *Biochimie*, 93, 5, (2011), 962-968 https://doi.org/10.1016/j.biochi.2011.02.006

[4] Hugo Fraga, Ricardo Graña-Montes, Ricard Illa, Giovanni Covaleda and Salvador Ventura, Association Between Foldability and Aggregation Propensity in Small Disulfide-Rich Proteins, *Antioxidants & Redox Signaling*, 21, 3, (2014), 368-383 https://doi.org/10.1089/ars.2013.5543

[5] J. M. Thornton, Disulphide bridges in globular proteins, *Journal of Molecular Biology*, 151, 2, (1981), 261-287 https://doi.org/10.1016/0022-2836(81)90515-5

[6] Shunsuke Kawamura, Mari Ohkuma, Yuki Chijiiwa, Daiki Kohno, Hiroyuki Nakagawa, Hideki Hirakawa, Satoru Kuhara and Takao Torikata, Role of disulfide bonds in goose-type lysozyme, *The FEBS Journal*, 275, 11, (2008), 2818-2830 https://doi.org/10.1111/j.1742-4658.2008.06422.x

[7] Tony A. Klink, Kenneth J. Woycechowsky, Kimberly M. Taylor and Ronald T. Raines, Contribution of disulfide bonds to the conformational stability and catalytic activity of ribonuclease A, *European Journal of Biochemistry*, 267, 2, (2000), 566-572 https://doi.org/10.1046/j.1432-1327.2000.01037.x

[8] Chih-Hsien Wang and Wenlung Chen, Raman Characterizing Disulfide Bonds and Secondary Structure of Bovine Serum Albumin, *AIP Conference Proceedings*, 1267, 1, (2010), 346-347 https://doi.org/10.1063/1.3482550

[9] Zoya Bumagina, Bella Gurvits, Natalya Artemova, Konstantin Muranov and Boris Kurganov, Paradoxical Acceleration of Dithiothreitol-Induced Aggregation of Insulin in the Presence of a Chaperone, *International Journal of Molecular Sciences*, 11, 11, (2010), 4556-4579 https://doi.org/10.3390/ijms11114556

[10] Jessica Gobbo, Caroline Gaucher-Di-Stasio, Stéphanie Weidmann, Jean Guzzo and Carmen Garrido, Quantification of HSP27 and HSP70 Molecular Chaperone Activities, in: S.K. Calderwood, T.L. Prince (Eds.) *Molecular Chaperones: Methods and Protocols*, Humana Press, Totowa, NJ, 2011, pp. 137-143 https://doi.org/10.1007/978-1-61779-295-3_11

[11] Mu Yang, Colina Dutta and Ashutosh Tiwari, Disulfide-Bond Scrambling Promotes Amorphous Aggregates in Lysozyme and Bovine Serum Albumin, *The Journal of Physical Chemistry B*, 119, 10, (2015), 3969-3981 https://doi.org/10.1021/acs.jpcb.5b00144

[12] Kazuo Hirayama, Satoko Akashi, Mami Furuya and Ken-ichi Fukuhara, Rapid confirmation and revision of the primary structure of bovine serum albumin by ESIMS and frit-FAB LC/MS, *Biochemical and Biophysical Research Communications*, 173, 2, (1990), 639-646 https://doi.org/10.1016/S0006-291X(05)80083-X

[13] Damien Hall, Ran Zhao, Ian Dehlsen, Nathaniel Bloomfield, Steven R. Williams, Fumio Arisaka, Yuji Goto and John A. Carver, Protein aggregate turbidity: Simulation of turbidity profiles for mixed-aggregation reactions, *Analytical Biochemistry*, 498, (2016), 78-94 https://doi.org/10.1016/j.ab.2015.11.021

[14] Ran Zhao, Masatomo So, Hendrik Maat, Nicholas J. Ray, Fumio Arisaka, Yuji Goto, John A. Carver and Damien Hall, Measurement of amyloid formation by turbidity assay—seeing through the cloud, *Biophysical Reviews*, 8, 4, (2016), 445-471 https://doi.org/10.1007/s12551-016-0233-7

[15] David J. Pulford, Debbie F. Frost, Dominic D. Lomiwes and Mustafa M. Farouk, Preliminary studies to determine the chaperoning properties of bovine casein and crystallin proteins at reducing beef muscle protein aggregation during heating, *International Journal of Food Science & Technology*, 43, 12, (2008), 2143-2150 https://doi.org/10.1111/j.1365-2621.2008.01821.x

[16] Stefan K. Baier, Eric A. Decker and D. Julian McClements, Impact of glycerol on thermostability and heat-induced gelation of bovine serum albumin, *Food Hydrocolloids*, 18, 1, (2004), 91-100 https://doi.org/10.1016/S0268-005X(03)00046-8

[17] Vera A Borzova, Kira A Markossian, Dmitriy A Kara, Natalia A Chebotareva, Valentina F Makeeva, Nikolay B Poliansky, Konstantin O Muranov and Boris I Kurganov, Quantification of anti-aggregation activity of chaperones: a test-system based on dithiothreitol-induced aggregation of bovine serum albumin, *PLoS One*, 8, 9, (2013), e74367 https://doi.org/10.1371/journal.pone.0074367

[18] Bill X. Huang, Hee-Yong Kim and Chhabil Dass, Probing three-dimensional structure of bovine serum albumin by chemical cross-linking and mass spectrometry, *Journal of the American Society for Mass Spectrometry*, 15, 8, (2004), 1237-1247 https://doi.org/10.1021/jasms.8b02235

[19] Parvez Alam, Ayesha Zainab Beg, Mohammad Khursheed Siddiqi, Sumit Kumar Chaturvedi, Ravi Kant Rajpoot, Mohd Rehan Ajmal, Masihuz Zaman, Ali S. Abdelhameed and Rizwan Hasan Khan, Ascorbic acid inhibits human insulin aggregation and protects against amyloid induced cytotoxicity, *Archives of Biochemistry and Biophysics*, 621, (2017), 54-62 https://doi.org/10.1016/j.abb.2017.04.005

[20] Jian-Bo Wang, Yi-Min Wang and Cheng-Ming Zeng, Quercetin inhibits amyloid fibrillation of bovine insulin and destabilizes preformed fibrils, *Biochemical and Biophysical Research Communications*, 415, 4, (2011), 675-679 https://doi.org/10.1016/j.bbrc.2011.10.135

[21] Zohra Dhouafli, Karina Cuanalo-Contreras, El Akrem Hayouni, Charles E. Mays, Claudio Soto and Ines Moreno-Gonzalez, Inhibition of protein misfolding and aggregation by natural phenolic compounds, *Cellular and Molecular Life Sciences*, 75, 19, (2018), 3521-3538 https://doi.org/10.1007/s00018-018-2872-2

[22] Min Zhu, Shubo Han and Anthony L. Fink, Oxidized quercetin inhibits α-synuclein fibrillization, *Biochimica et Biophysica Acta (BBA) - General Subjects*, 1830, 4, (2013), 2872-2881 https://doi.org/10.1016/j.bbagen.2012.12.027

[23] Palak Patel, Krupali Parmar, Dhaval Patel, Suresh Kumar, Manan Trivedi and Mili Das, Inhibition of amyloid fibril formation of lysozyme by ascorbic acid and a probable mechanism of action, *International Journal of Biological Macromolecules*, 114, (2018), 666-678 https://doi.org/10.1016/j.ijbiomac.2018.03.152

[24] Megha Bansal, Neha Singh, Saurabh Pal, Indra Dev and Kausar Mahmood Ansari, Chapter Three - Chemopreventive Role of Dietary Phytochemicals in Colorectal Cancer, in: J.C. Fishbein, J.M. Heilman (Eds.) *Advances in Molecular Toxicology*, Elsevier, 2018, pp. 69-121 https://doi.org/10.1016/B978-0-444-64199-1.00004-X

[25] Andrea Baschieri, Riccardo Amorati, Tiziana Benelli, Laura Mazzocchetti, Emanuele D'Angelo and Luca Valgimigli, Enhanced Antioxidant Activity under Biomimetic Settings of Ascorbic Acid Included in Halloysite Nanotubes, *Antioxidants*, 8, 2, (2019), 30 https://doi.org/10.3390/antiox8020030

[26] Lu-Fei Yang and Cheng-Ming Zeng, The Degradation Products of Ascorbic Acid Inhibit Amyloid Fibrillation of Insulin and Destabilize Preformed Fibrils, *Molecules*, 23, 12, (2018), 3122 https://doi.org/10.3390/molecules23123122