SPECTROSCOPIC CHARACTERIZATION OF BAKER YEAST ALDEHYDE DEHYDROGENASE DURING BINDING OF NAD(P)$^+$ AND SOME POLYPHENOLS

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Abstract. Aldehyde dehydrogenase (ALDH) is a superfamily of phase I oxidizing enzymes responsible for detoxification of biogenic and xenogenic aldehydes to the corresponding carboxylic acids by means of an NAD(P)$^+$-dependent reaction. The enzyme has been implicated in the protection of prokaryotic and eukaryotic organisms against various oxidative stress conditions. In this study, we investigated into the preferred coenzyme used by the enzyme in carrying out its function at different solution pH and The binding properties of NAD^+ and $NADP^+$, rutin, tannic acid, quercetin and gallic acid to aldehyde dehydrogenase was investigated at 25 ºC (298 K) and pH 9.0 by UV-Vis absorption spectroscopy and fluorescence quenching spectroscopy. The specific binding constants of NAD^+ and $NADP^+$ to the enzyme are 1.50×10^5 M^{-1} and 1.58×10^5 M^{-1}, respectively. This demonstrates ALDH preference for $NADP^+$, though dual specificity cannot be ruled out; however, the dissociation of the coenzymes from the enzyme was pH dependent. The fluorescence spectra of the interaction of rutin, gallic acid and quercetin with ALDH showed static quenching mechanism while gallic acid was dynamic quenching. All with stoichiometric ratio of one to ALDH. Gallic has the least quenching efficiency. The dissociation of NAD^+ from ALDH was also found to be dependent on the viscosity of the solution using glycerol as micro-viscogen and ficcol 400 as macro-viscogen.

Key words: Spectroscopic techniques, aldehyde dehydrogenase, coenzymes, NAD(P)$^+$, ligands.

INTRODUCTION

Aldehyde dehydrogenases (ALDHs; EC 1.2.1.3) are superfamily of NAD(P)$^+$-dependent oxidoreductases involved in the oxidation and detoxification of a wide range of physiological and pathophysiological aldehydes to their corresponding carboxylic acids and therefore protects cells against aldehyde induced oxidative stresses [8, 15, 24]. The ALDH superfamily is comprised of 19 human isoenzymes subdivided into 11 families and 4 subfamilies. Many of the ALDH isoenzymes overlap in relation to subcellular localization (cytoplasm,

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mitochondria, or nucleus), tissue distribution, and substrate specificities but vary in their efficiency in metabolizing specific aldehydes [8, 15]. Oxidation of these reactive aldehydes to their corresponding carboxylic acids, a major detoxifying pathway of aldehydes, occurs through the use of NAD^+ or $NADP^+$ as cofactor [24]. In mammals, the role of ALDH in the biosynthesis of retinoic acid (RA), γ-aminobutyric acid, and betaine for regulation of cellular homeostasis has been documented [7, 11]. The enzyme has been identified as metabolic stem cell marker [12, 24]. The human ALDHs are involved in several regulatory functions including cell proliferation, neurotransmission, biosynthesis, osmoregulation, embryogenesis, development, carcinogenesis and drug resistance [7, 8, 11, 12, 15, 24]. ALDH up-regulation occurs in bacteria in response to environmental and chemical stressors; in plants in response to dehydration, salinity, and oxidative stress; in yeasts after exposure to ethanol and oxidative stress; and in mammals in response to oxidative stress and lipid peroxidation [21]. The ability of ALDHs to act as ‘‘aldehyde scavengers’’ is grounded in the observation that they have broad substrate specificities and can metabolize a wide range of chemically and structurally diverse aldehydes [22]. Aldehydes are strong electrophilic, highly reactive compounds which readily form adducts with deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and proteins, leading to impaired cellular homeostasis, enzyme inactivation, DNA damage, and cell death [22]. The oxidative stress generated by these radicals are usually combatted by the utilization of plants flavonoids, a large group of polyphenolic natural products are increasingly being recognized as possessing a broad spectrum of biological activities including oxidant or free radical protection, ultraviolet radiation protection, modulation of enzyme activity, allelopathy acting as anti-cancer, anti-inflammatory and anti-coagulant agents [7, 22]. Interestingly, many of these biologically active flavonoids appear to have effects on various proteins, including enzymes [3]. ALDHs family is similar and structurally conserved consisting of catalytic, co-enzyme binding and oligomerization domains [23]. The coenzyme binding domain, a variant of the Rossman fold, essential for coordinating NAD^+ and $NADP^+$-helices [9]. ALDH can be either NAD^+ specific, $NADP^+$ specific or dual in ALDH. The domain is composed of five β-strands connected by four α-coenzyme specific [14]. However, a few site directed mutations can reverse this coenzyme specificity. The specific binding of these coenzymes to ALDH is mediated by selective interactions between different enzyme nucleotide amino acid residues and different parts of the coenzymes [18]. Specificity for NAD^+ or for $NADP^+$ has probably emerged repeatedly during evolution, using different structural solutions on different occasions [18]. This study is designed however, to reveal the type of interaction between the coenzymes $NAD(P)^+$, some polyphenols and the enzyme, ALDH during catalysis and to show the preferential coenzyme. The conformational changes in the structure of the protein upon the binding of the coenzymes and flavonoids were also studied.

MATERIALS AND METHODS

MATERIALS

Baker's yeast aldehyde dehydrogenase (ALDH) (Cat #82884), nicotinamide adenine dinucleotide (NAD^+) (Cat #N0632), nicotinamide adenine dinucleotide phosphate ($NADP^+$) (Cat #5755), potassium phosphate monobasic (Cat #5655), 1,8-anilinonaphtalenesulphonic acid (Cat #A1082), glycine (Cat #G8898), sodium acetate (Cat #2889), di-potassium hydrogen phosphate (Cat #3786) trizma base (Cat #T1503), ethanol (Cat #32205) acetic acid (Cat #320099), quercetin (Cat #Q4951), rutin (Cat #5143), gallic acid (Cat #G7384), tannin acid (Cat #403040) and ficcol 400 (Cat #F4512) were acquired from Sigma Aldrich Fine Chemicals, St. Louis, MO, USA.

TITRATIONS OF ALDH WITH NAD^+ AND $NADP^+$

General titration methods and analysis as previously described by Royal *et al*. [20] was adopted. Stock solutions of coenzymes NAD^+ and $NADP^+$ (10 mM) were prepared in 25 mM Tris-HCl buffer (pH 8.0) were used without adjusting the pH. All absorbance spectra and equilibrium ligand binding experiments were measured in 25 mM Tris-HCl buffer, pH 8.0, at 25 ^oC using Shimadzu double beam UV-Visible spectrophotometer (UV-1800) equipped with a Pharmacia refrigerating circulator for temperature control (25 ± 0.1 ^oC) unless otherwise, stated and interface to Hp Window XP Computer. The scan speed and slit of absorbance (λ_{abs}) were set to medium and 1.0 nm respectively. The sampling interval was adjusted to 0.5 nm and scan mode single. The spectra were recorded between 200–500 nm. Equilibrium binding constant, K_a, for binding of NAD^+ and $NADP^+$ was determined according to published procedures of Basran *et al*. [2]. Equilibrium binding constant, K_a, were determined spectrophotometrically by monitoring the change in absorbance at the appropriate wavelength and fitting to equation (1):

$$\Delta\text{abs} = \left(K_a A_i + [L]_{tot} A_f\right) / \left(\left(K_a [L]_{tot}\right)\right) \quad (1)$$

where A_i and A_f are the initial and final absorbance values, respectively, and $[L]_{tot}$ is the total concentration of the either coenzymes. The binding of the coenzymes to the enzyme was followed at the highest peaks at far UV region. The dissociation constants were calculated as described elsewhere [4].

pH TITRATIONS FOR TH E SPECTRA CHANGE OF ALDEHYDE DEHYDROGENASE WITH NAD(P)$^+$

The pH dependence of the absorption spectrum of the aldehyde dehydrogenase in the presence of NAD^+ or $NADP^+$ was examined at 25 mM glycine-HCl buffer pH 3.0; 25 mM sodium acetate buffer at pH 4.0 and 5.0, 25 mM phosphate buffer at pH 6.0 and 7.0 and 25 mM Tris-HCl buffer at pH 8.0 and 9.0. The binding constant, K_a and dissociation constant, K_d were evaluated as described earlier.

FLUORESCENCE QUENCHING

A quantitative analysis of the potential interaction between NAD^+, $NADP^+$, rutin, quercetin, gallic acid and tannic with aldehyde dehydrogenase was carried out by fluorimetric titration. quercetin, rutin, gallic acid and tannic acid were each dissolved in 50 % absolute ethanol and filtered with 0.22 μm filter (VWR, Radnor, PA, USA). All fluorescence spectra were recorded on F-4500 Spectrofluorimeter (Hitachi, Japan) equipped with a thermostat bath interfaced with HP Window XP Computer. Exactly 2.0 mL solution containing 5 μM aldehyde dehydrogenase was added to a 1.0 cm fluorescence quartz cuvette, and then titrated by successive addition of the ligand solution with a cuvette mixer. These solutions were allowed to stand for 1 min to equilibrate, and then the fluorescence spectra were measured at 298 K in the wavelength range of 300–450 nm upon exciting wavelength at 280 nm. The widths of both the excitation slit and emissions slit were set at 5.0 nm at a scanning speed of 1200 nm/min and 700 V was used and the response time was set to be 0.01 sec. The appropriate blanks corresponding to the Tris-HCl buffer solution were subtracted to correct background of fluorescence. The intensity of fluorescence used was the corrected fluorescence intensity. The operative quenching mechanism in the coenzyme, polyphenols-ALDH systems was deduced from the Stern-Volmer equation:

$$F_0 / F = 1 + K_{SV}[Q] = 1 + K_q\tau_0[Q] \quad (2)$$

where F_0 and F are the fluorescence intensities of aldehyde dehydrogenase in the absence and presence of quencher, respectively. K_q is the quenching rate constant of biomolecule, τ_0 is the average lifetime of biomolecule without quencher and the value of τ_0 of biopolymer is 10^{-8} s. K_{sv} is the Stern-Volmer dynamic quenching constant, and $[Q]$ is the concentration of quencher. The K_{sv} at 25 ^{o}C (298 K) was determined by linear regression plot of F_0/F versus $[Q]$. Furthermore, the apparent

binding constant (K_a) and number of bound ligand/coenzyme to aldehyde dehydrogenase (n) were determined by plotting the double logarithm regression curve using the following equation:

$$\mathrm{Log}\frac{F_0 - F}{F} = \mathrm{Log}K_a + n\mathrm{Log}[Q] \tag{3}$$

The association constant was estimated by the modified Stern-Volmer equation

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a K_a}\frac{1}{[Q]} + \frac{1}{f_a} \tag{4}$$

where K_a is the modified Stern-Volmer association constant for the accessible fluorophores, and f_a is the fraction of accessible fluorescence. From the plot of $F_0/(F_0 - F)$ versus $1/[Q]$, the values of K_a and f_a were obtained from the values of slope and intercept, respectively. The presence of this volume of ethanol in the assay mixtures had no effect on the fluorescence measurements. Also, respective blanks of the buffer were used for the correction of all fluorescence spectra. The experiments were repeated and found to be reproducible within experimental errors. However, the fluorescence intensity was corrected using the equation 5, in order to eliminate the inner filter effects of ALDH and polyphenols:

$$F_{cor} = F_{obs} \times e^{(A_1 + A_2)/2} \tag{5}$$

where, F_{cor} and F_{obs} are the fluorescence intensity corrected and observed, respectively; A_1 and A_2 are the sum of the absorbances of ALDH and polyphenol at excitation and emission wavelengths, respectively. All fluorescence spectra are corrected for the absorption of quencher at the excitation and emission wavelengths of the fluorophore. The maximum emission intensities were used to calculate binding constants, number of binding site and thermodynamic parameters.

EFFECT OF VISCOGEN ON K_d OF NAD^+-ALDH COMPLEX

Experiments to probe the effects of solution viscosity upon equilibrium dissociation constant, K_d, were conducted using glycerol as micro-viscogen and ficcol 400 as macro-viscogen in order to detect non-specific effects arising from ALDH structural perturbation as described by Huff *et al.* [6]. Intrinsic and extrinsic viscosities were determined relative to a solution containing only buffer using an Ostwald viscometer at 25 °C. The resulting data were fitted to:

$$\left[K_{\mathrm{d}}\right]^{0} / \left[K_{\mathrm{d}}\right] = \left(\eta^{\mathrm{rel}}/\eta^{0}\right)^{exp} \tag{6}$$

where the superscript '0' designates the absence of added viscogen. According to the Stokes-Einstein relation, an exponent (*exp*) of 1 is expected for diffusion-limited binding of the NAD^+ to ALDH. A smaller empirically determined exponent of lower than 0.5 has been proposed to describe the deviations from Stokes-Einstein behavior [19].

DATA ANALYSIS

Experimental data were expressed as mean ± SD of the representative experiments; experiments were repeated at least thrice. Statistical analysis was performed using a two tailed student's t-test or analysis of variance (ANOVA) followed by the two tailed Dunnett's test. p values ≤ 0.05 are to be considered as significant. KaleidaGraph 4.0 (Synergy software, USA) software was used to test the significance of the experiment and for Graphical analysis.

RESULTS AND DISCUSSIONS

The result presented by the UV-Vis absorption spectra reveals a change in the ALDH absorption spectra between 200 nm and 500 nm upon interaction with various concentrations of coenzymes NAD^+ and $NADP^+$. The UV-Vis absorption spectra (200–500 nm) of ALDH in the presence of coenzymes $NADP^+$ and NAD^+ at different concentrations is shown in Figs 1 and 2.

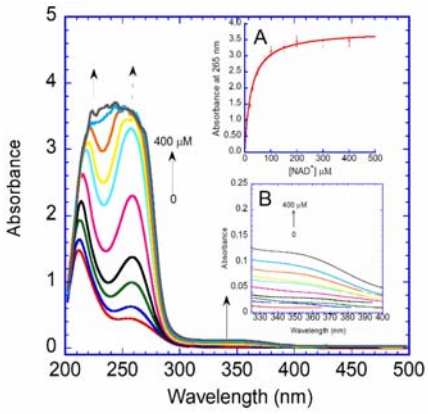

Fig. 1. Electronic absorption spectra of 5.0 µM aldehyde dehydrogenase (ALDH) in the presence of NAD^+ (0–400 µM) from 200 nm to 500 nm. Reaction conditions: 25 mM Tris-HCl, pH 9.0, 25.0 ºC. Inset A. The saturation curve of the absorption spectra at 265 nm and Inset B is the spectra between 320–400 nm.

ALDH-NAD(P)$^+$ spectra exhibited three absorption peaks. One, the peak at 210 nm that reflected the n–π* transition of the polypeptide backbone of ALDH upon interaction with the various concentrations of the coenzymes. The second peak at 256 nm is connected to the π–π* transition of the ALDH intrinsic aromatic amino acid residues during complexation with the coenzymes [1]. The third peak, though not very conspicuous, is at 350 nm. An increase in the concentration of NAD(P)$^+$ increase the intensity of the ALDH-NAD(P)$^+$ complex UV-Vis causing the absorption peak at 210 nm in far UV region to causing a redshift (210 nm → 214 nm).

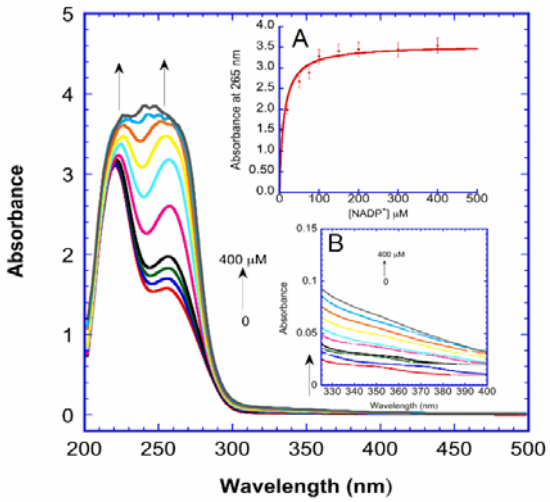

Fig. 2. Electronic absorption spectra of 5 µM aldehyde dehydrogenase (ALDH) in the presence of NADP$^+$ (0–400 µM) from 200 nm to 500 nm. Reaction conditions: 25 mM Tris-HCl, pH 9.0, 25.0 °C. Inset A. The saturation curve of the absorption spectra at 265 nm and Inset B is the spectra between 320–400 nm.

This indicated perturbation around the peptide bonds causing slight increase in size of the complex which could orientate the complex to favor the binding of aldehydic substrates. However, there was blue shift of 8 nm from 256 nm to 248 nm in the presence of saturating concentration of 400 µM NAD$^+$ (Fig. 2) and also a blue shift of 4 nm (255 nm to 250 nm) in the presence of saturating concentration of NADP$^+$ (Fig. 3) causing the global aromatic hydrophobic amino acids of ALDH to be more compact indicating a possible interaction, conformational changes and the dependence of ALDH on the nucleotide molecules as co-enzymes in agreement with the report of Lo *et al.* [10] that ALDH is an NAD(P)$^+$-dependent enzyme. The result suggests that as the coenzymes are being complexed with the protein, the amino acid microenvironment was made more compact and there was an induction of conformation change on the enzyme which enabled it to attain equilibrium saturation. The specific binding of complex molecules such as NAD$^+$ or NADP$^+$ to

dehydrogenases is mediated by selective interactions between different enzyme amino acid residues and different parts of the dinucleotide molecules [10]. The saturating plots of the ALDH-NAD(P)$^+$ at 350 nm is shown in Fig. 2 (inset).

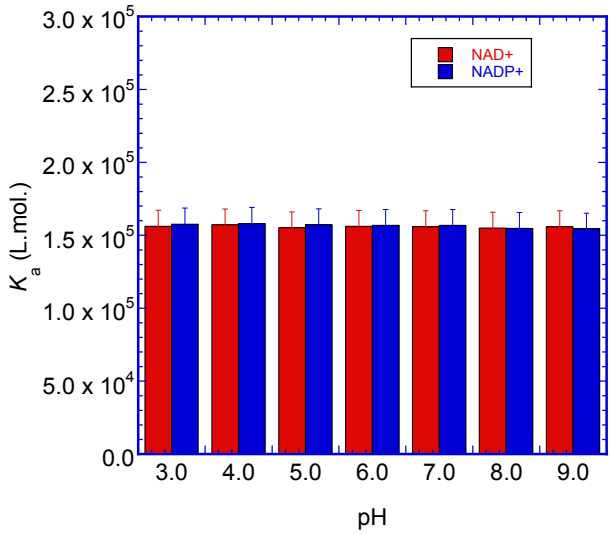

Fig. 3. Association constant plot of (0–400 µM) NAD$^+$ and NADP$^+$ with 1.0 mL of 5 µM aldehyde dehydrogenase under varied pH condition. Reaction conditions: pH 3, 25 mM glycine buffer, pH 4 and 5, 25 mM sodium acetate buffer, 25 mM phosphate buffer, and pH 8 and 9, 25 mM Tris-HCl buffer.

The plot is the changes in absorbance at 350 nm with respect to increase in concentrations of NAD(P)$^+$ and affinity factor, K_M, was evaluated. This subtly demonstrate that NADP$^+$ could be preferential coenzyme for ALDH compared to NAD$^+$. The affinity factor for NADP$^+$ was slightly lower than NAD$^+$. However, the dual coenzyme specificity cannot be ruled out. The exploration of change in the tertiary conformation of protein via the absorption spectra shows the conformation of protein structure from 356 nm to 354 nm in the presence of NAD$^+$ while interaction with NADP$^+$ indicates a slight conformational change from wavelength 354 nm to 353 nm indicating blue shift and slight conformational change with NAD$^+$ enhancing the compatibility of the protein more than NADP$^+$. The spectra suggest that less binding energy will be required for the alteration in the protein conformation in the presence of NAD$^+$ as the conformational change was observed at a wavelength higher than that of the NADP$^+$/ALDH. The pH-dependence measurement of association constants K_a and dissociation constants K_d was carried out from the ALDH-NAD(P)$^+$ absorption spectra between pH 3.0 and 9.0. The values obtained for the association constant remained almost unchanged throughout the pH under study (Fig. 4).

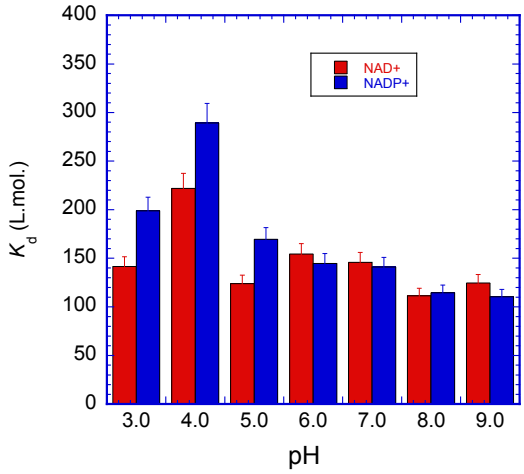

Fig. 4. Dissociation constant K_d plot of (0–400 µM) NAD^+ and $NADP^+$ with 1.0 mL of 5 µM aldehyde dehydrogenase under varied pH condition. Reaction conditions: (pH 3.0, 25 mM glycine buffer, pH 4.0 and 5.0, 25 mM sodium acetate buffer, 25 mM phosphate buffer, and pH 8.0 and 9.0, 25 mM Tris-HCl buffer).

This indicates that the binding specificity, K_a of NAD^+ and $NADP^+$ to ALDH was not pH dependent. However, the values of K_d varies with changes in pH and having highest K_d at acidic pH of 4.0. The K_d values of NAD^+ are higher compared $NADP^+$ at acidic pH but were converse at alkaline pH. The values have clearly demonstrated that K_d not K_a of ALDH-$NAD(P)^+$ complex determine of rate of ALDH catalysis and therefore the optimum pH for the catalysis is 9.0 for $NADP^+$ but 8.0 for NAD^+.

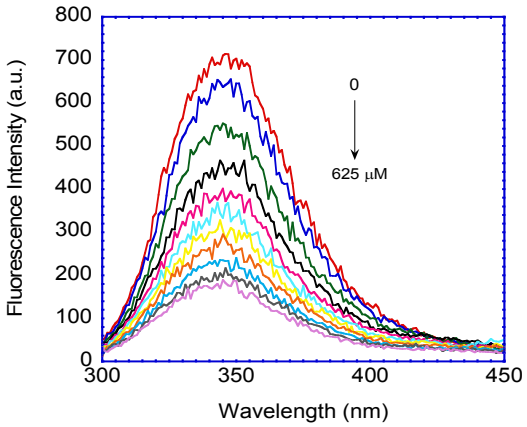

Fig. 5. Fluorescence emission spectra of rutin with ALDH (5.0 µM). 2.0 mL of 5 µM ALDH was titrated with successive addition of 62.5 µM rutin. Reaction condition: 25 mM Tris-HCl buffer (pH 9.0).

Living organisms are continuously being confronted with oxidative stress culminating from numerous reactive oxygen species (ROS) generated from metabolic activities on a regular basis. As a result, a research on antioxidant enzymes and many allelochemicals such as polyphenols having antioxidant properties has been on for years. This study, however, seeks to understand how these antioxidant compounds interact with and affect certain enzymes, in this case, aldehyde dehydrogenase (ALDH), an enzyme known to be $NAD(P)^+$-dependent. The binding effect of some polyphenols (gallic acid, rutin, quercetin and tannic acid) to ALDH, a target enzyme of the antioxidants is observed in the spectra change of the protein. The spectra change for rutin is shown in Fig. 5. A blue shift of 8 nm peak difference which was observed in the presence of rutin and quercetin correlates with the blue shift observed in the presence of $NAD(P)^+$. However, gallic acid and tannic acid showed red shift indicating the complex is made less compact. The quenching profile obtained for rutin, quercetin, and tannic acid shows that the quenching follows linear dependence on the quencher concentration indicating collisional mechanism of quenching.

The value of linearity (0.99) determined for rutin, quercetin, and tannic acid indicates that the quenching is either collisional or static while the extent of linearity which is below 0.99 obtained for gallic acid suggests a blue shift of 8 nm peak difference which was observed in the presence of rutin and quercetin correlates with the blue shift observed in the presence of $NAD(P)^+$. However, gallic acid and tannic acid showed red shift indicating the complex is made less compact. The quenching profile obtained for rutin, quercetin, and tannic acid shows that the quenching follows linear dependence on the quencher concentration indicating collisional mechanism of quenching. The value of linearity (0.99) determined for rutin, quercetin, and tannic acid indicates that the quenching is either collisional or static while the extent of linearity which is below 0.99 obtained for gallic acid suggests that the quenching mechanism may be combined mechanism of quenching as the curve plot obtained in the presence of gallic acid. The calculated values of biomolecular quenching constants (K_q) were 5.0×10^{12} $M^{-1}s^{-1}$, 2.3×10^{12} $M^{-1}s^{-1}$, 3.0×10^{12} $M^{-1}s^{-1}$ and 1.2×10^{10} $M^{-1}s^{-1}$ for gallic acid, rutin, quercetin and tannic acid, respectively. The calculated apparent bimolecular quenching constant, K_q, were of the order of 10^{12} M^{-1} s^{-1} based on Eq. 1. The values were 100 times lower than the maximum permissible value for dynamic quenching i.e. 2×10^{10} $M^{-1}s^{-1}$ except for tannic acid. Modified Stern-Volmer plots obtained for all four flavonoids from which fractional accessibility (f_a) was obtained and listed in Table 1. Based on the fa values of the quenchers used, 40 % and 45 % of the total fluorescence was found to be accessible to gallic acid, and tannic acid respectively, and 54 % and 4 % was accessible to quercetin and rutin respectively indicating an access to some tryptophan residues during interaction.

Table 1

Calculated Stern-Volmer quenching constants, apparent binding constants, apparent number of bound ligand, Stern-Volmer association constant, and the fraction of accessible fluorophore of the interaction between 5 μM ALDH and the polyphenols

Ligands	K_{sv} **(Lmol^{-1})**	K_q**(×10^{12})** **(Lmol^{-1}s^{-1})**	**(K_a) (Lmol^{-1})**	***n***	F_a
Gallic acid	5.0×10^4	5.0×10^{12}	4.48×10^3	1.11	0.404
Rutin	2.3×10^4	2.3×10^{12}	1.26×10^3	1.17	0.047
Quercetin	3.0×10^4	3.04×10^{12}	1.95×10^3	1.15	0.540
Tannic acid	1.2×10^2	2.7×10^{10}	4.5×10^3	0.94	0.454

K_{sv} = Stern-Volmer dynamic quenching constant, K_q = quenching rate constant of biomolecule, K_a is the association constant, n = number of binding site and F_a = fraction of accessible fluorophore.

The single binding sites observed from the interaction between the protein and the flavonoids is in accordance with the work done by Pastukhov *et al*. [17] which indicates the discovery of single binding site in an interaction between rutin, a flavonol and human serum albumin. The Stern-Volmer plots for the fluorescence quenching of the protein indicates that quenching profile obtained for the protein with tannin, rutin and quercetin which follows linear dependence on the quencher concentration indicates linear type of quenching while the profile obtained for gallic acid exhibit curve plot showing that certain tryptophan residues are quenched before others in the protein molecule. The linear mechanism of quenching exhibited for rutin, quercetin, and tannic acid is in correlation with investigation of Pastukhov *et al.* [17].

Studies on the dissociation of the coenzyme NAD^+ from ALDH as a function of solution viscosity shows that the relative dissociation constants of the NAD^+ from the ALDH decreased slightly with increase in the relative solution viscosities in the presence of added glycerol as micro-viscogen and ficcol 400 as macro-viscogen. The experiments carried out at high relative solution viscosities ($\eta^{rel} = 5$) showed that rate constant is indistinguishable for solutions containing up to 40 % glycerol and 30 % ficcol 400 in spite of the fractional (~0.5) exponent (Fig. 6). However, no viscosity effect is discernible on the dissociation constant of NAD^+ when the coenzyme is present at near-saturating concentrations irrespective of the viscogen which is in agreement with earlier studies on the effect of viscosity on the ligand binding and catalytic efficiencies of some proteins that the binding and dissociation of ligands to and from the binding site of the protein is dependent on the viscosity of the medium [13, 16].

CONCLUSIONS

This spectroscopic characterization has demonstrated the dual co-enzyme specificity for the Baker yeast aldehyde dehydrogenase with indistinguishable preference for $NADP^+$. The dissociation of the ALDH-$NAD(P)^+$ complex rather

than the association is the rate limiting step in the catalytic functionality of the enzyme. This could be regulated by change in pH and environment viscosity. ALDH has stoichiometric ratio of one for its coenzyme and other polyphenols used in this study.

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