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A STUDY ON THE EQUILIBRIUM BINDING OF KOJIC ACID WITH β-AMYLASE.

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ABSTRACT
The binding interaction of kojic acid with β-amylase was studied under simulative conditions using fluorescence spectroscopy. The results obtained revealed kojic acid quenched intrinsic fluorescence of β-amylase to form β-amylase-kojic acid complex. The quenching was a static quenching with effective quenching constant (K_{sv}) of 2.13 X 10^2 L. mol^{-1}. The Scatchard plot indicated that β-amylase has one binding site for Kojic acid with association constant of 1.55 x 10^3 L. mol^{-1} at 298 K and pH 7.4. The bonding represents a favourable bonding interaction. With these, β-amylase as a nano-vehicle for kojic acid is hereby suggested.
Keywords: Kojic acid; β-amylase; Fluorescence; Quenching.

INTRODUCTION
β-Amylase [E.C. 3.2.1.2 α-1,4,-D-glucan maltohydrolase] plays an important role in the biogeochemical cycle of carbon and it also has a wider application in the biotechnological-based pharmaceutical, food and beverage industries in the conversion of starch into maltose solution (Ziegler, 1999). -amylase hydrolyses α-1,4-glucosidic linkages of the starch-type substrate in an exo-fashion from the non-reducing end to β-maltose (Visko-Neilsen *et al*, 1997). Substrates for the enzyme include α-(1,4)-glucans e.g. amylose, amylopectin and limit dextrins with a minimal chain length of four glucose units (Lizotte *et al*. 1990). β-Amylase is found in microorganism (bacteria, fungi) and plants but absent in animal tissues. In plants, β-amylase occurs in free and combined forms (Evans *et.al*, 1997). It has been isolated and studied extensively in different plant species. β-Amylase has previously been purified and characterized from sweet potatoes, soybean, barley, rye, and pea (Brena *et al*, 1996). This enzyme together with other hydrolytic enzymes are said to be involved in the breakdown of starch during germination of seeds (Guglieminetti *et al.*, 1995) and in the development of its embryo. β-amylase is synthesized during grain development and stored in mature grains.

Kojic acid, 5-hydroxy-2-hydroxymethyl-4-pyrone, (Fig 1) is produced by several species of fungi, especially *Aspergillus oryzae*. It is a natural antibiotic agent obtained from Koji malt (*Aspergillus oryzae*). Koji malt has been used for the production of miso, soya sauce and sake in Japan for a long time (Budavari, 2000). Kojic acid can act as a tyrosinase inhibitor (to inhibit melanin

formation), an antioxidant, a bacteriostat, a metal chelating agent and an intermediate in synthesis.

The kojic acid is produced mainly from carbohydrate sources in an aerobic process by a variety of microorganisms. Kojic acid is used in the prevention of discolouration of crustacea, meat and fresh vegetables; as a preservative, as an antioxidant for fats and oils (Burdock *et al.,* 2001) in cosmetics (skin whitening or depigmenting agent), in the preparation of derivative esters (i.e. kojic oleate, kojic stearate), in adhesives, in chelate-forming resins and as a plant growth-regulating agent to increase production, early maturing and increase sweetness (Cabanes *et al.*, 1994; Alverson, 2003). Kojic acid is an important group of classical inhibitors form of compounds structurally analogous to phenolic substrates, towards which they generally show competitive inhibition, although this inhibition varies depending on the enzyme source and substrate used (Aytemir and Ozcelik, 2011).

Although a vast number of β-amylase have been studied exclusively with only few reports on its binding properties. The binding of kojic acid to β-amylase using fluorescence spectroscopy has not been previously reported.

MATERIALS AND METHODS

Materials

β-amylase, Kojic acid, 3-(N-morpholino)propanesulfonic acid (MOPS), 2-(*N*-morpholino)ethanesulfonic acid (MES), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES), Sodium chloride (NaCl) , Ethanol were purchased from Sigma-Aldrich Fine Chemicals, St. Louis, MO, USA. Bradford reagent and kit were products of BioRad, Palo Alto, CA, USA. β-amylase was purified to apparent homogeneity by α-cyclodextrin-Sepaharose 6B affinity chromatography column (Totsuka and Fukazawa, 1993). The homogeneity was confirmed on 12 % SDS-PAGE. All other chemicals were commercial products of analytical grade unless specialized. All solutions were prepared with doubled distilled water. β-amylase protein concentrations were measured by Bradford method (Bradford, 1976). Protein sample, ligand solutions and buffers were filtered through a Millipore membrane filter (0.45 micron syringe filters) immediately before use.

Determination of β-amylase-Kojic Acid binding constant.

Hitachi F-4500 fluorescence spectrometer (Hitachi Ltd., Tokyo, Japan) interfaced to a refrigerated circulating water bath (Pharmacia Biotech, Uppsala, Sweden) and 1.0 cm quartz cell was used to obtain the fluorescence emission spectra. Fluorescence emission data were stored at 10 Hz sampling rate to a Dell PC (Windows XP). The equipment was furnished with a 150 W Xenon lamp. The spectra were recorded in the wavelength range of 300–500 nm upon excitation at 280 nm when β-amylase samples were titrated with the ligand (kojic acid). Both excitation and emission bandwidths were set on 5 nm with a scan speed at 900nm/min with a high sensitivity and a response time of 2 sec. Titrations were performed manually by using trace syringes. A 2.0 ml solution containing an appropriate concentration of β-amylase (0.250 μM) in 25 mM MOPS pH 7.4 containing 0.1M NaCl was titrated manually by successive additions of stock solution of kojic acid to final concentration of 87.5 μM. 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 quenching effect on the enzyme was investigated at its maximum emission range (340– 350 nm). The experiments were repeated in triplicate and found to be reproducible within the limits of experimental errors. The maximum emission intensities were used to calculate binding constants, occupation of binding sites and thermodynamic parameters. All UV-Vis absorbance spectral determinations were performed on a Shimadzu double beam UV-

Visible spectrophotometer (UV-1800) equipped with a Pharmacia refrigerating circulator for temperature control. The fluorescence quenching constant was analyzed using the Stern-Volmer equation (Deng and Liu, 2012).

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

$k_q = k_{SV}/\tau_0$ (2)

Where, F_0 and F are fluorescence intensities before and after the addition of the quencher (Kojic acid) respectively. k_{SV} is the Stern–Volmer quenching constant, [Q] is the concentration of the quencher (Kojic acid), k_q is the quenching rate constant of biomolecule and it is equal to k_{sv} / τ_0. τ_0 is the average lifetime of the biomolecule in the presence of the quencher ($\tau_0 = 10^{-8}$s). Investigation of the binding constant of the ligand on the protein and the number of binding sites were achieved using Scatchard analysis:

$$log\ \frac{F_0-F}{F} = log\ k_a + n\ log\ [Q] \quad (3)$$

Where K_a is the binding/quenching constant of interaction between the quenching agent and the protein, n is the number of binding sites.

Fluorescence life-time studies

The fluorescence quenching data were analyzed by the mathematical model of Stern-Volmer equation (Eqns. 1 and 2) to determine the flourescence life time measurements as an indicator for the Binding/\Quenching mechanism.

Statistical and Graphical Analysis

Statistical and graphical analysis was done using the Kaleida Graph 4.5 (Synergy Software, Reading PA, USA) for Macintosh Computer.

RESULTS AND DISCUSSION

Analysis of fluorescence Quenching of β-amylase by Kojic acid.

Fluorescence quenching techniques is a veritable approach to measure binding affinity and measure the decrease of the quantum yield of fluorescence from a fluorophore induced by a variety of molecular interactions with quencher molecule (Oravcova et al, 1996). It has been exploited to examine proteins structural and functional properties, and protein-ligand interaction (Royer, 2006). The change in the intrinsic fluorescence of β-amylase measured as a function of kojic acid concentration (0-87.5μM) at 298K and pH 7.4 after excitation at 280 nm is shown in Fig 2. The natural fluorescence emission spectrum of β-amylase displays a single peak with a maximum at 346 nm. The maximum emission peak at 346 nm (less than 350 nm) inferred that the β-amylase intrinsic fluorophores were relatively buried. The intrinsic fluorescence was gradually quenched with successive addition of the kojic acid. The fluorescence emission intensity of β-amylase decreased consistently with the successive equilibrium addition of kojic acid. This suggests kojic acid could interact with β-amylase and quenched the enzyme intrinsic fluorescence to form a non-fluorescent β-amylase-kojic acid complex. This quenching revealed the accessibility of the enzyme intrinsic fluorophores to the ligand. The fluorescence spectra peak, shape and intensity were very stable and highly repeatable in the buffer used. Apparently, from the spectra, there were no blue nor red shifts of the emission maxima with the addition of the Kojic acid. The consistent maximum emission wavelength suggests there is no obvious change for the hydrophobicity of the microenvironment encompassing the β-amylase chromophore. Intriguingly, β-amylase-kojic acid complex formation might affect the antioxidant capacity of the kojic acid.

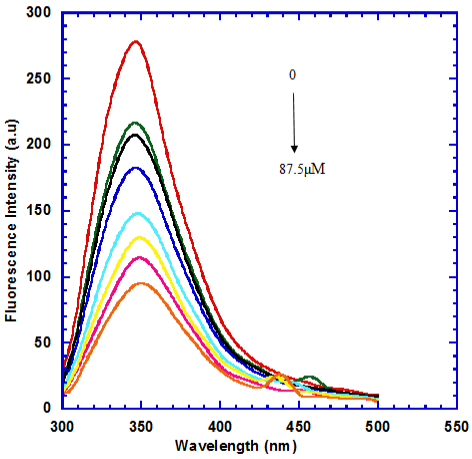

Figure 1: Fluorescence emission spectra of β-amylase-Kojic acid complexes in the absence and presence of different concentration of Kojic acid

Determination of the fluorescence quenching mechanism

Fluorescence quenching can be dynamic which results from collisional encounters between the fluorophore and quencher, or static, owing to the formation of a ground state complex between the fluorophore and quencher (Hu *et al*, 2005). The quenching rate constants decrease with increasing temperature for static quenching, but the reverse effect is observed for dynamic quenching. In general, static and dynamic quenching can be distinguished from their different binding constants dependent on temperature and viscosity, or preferably by lifetime measurements (Hu *et al*, 2005). The linear plot (Fig. 3) showed that only a single quenching mechanism was involved which could be static or dynamic quenching. The positive deviation of the plot would have demonstrated the simultaneous presence of both static and dynamic quenching. The results obtained from Stern-Volmer graph showed that the Stern-Volmer constant (K_{sv}) value is 2.13 X 10^2 L. mol^{-1}, which indicated that the probable quenching of β-amylase by kojic acid was strong. According to equation (2) above, the K_q values was calculated as 2.13 x 10^{10} L. mol^{-1} s^{-1}. The value was slightly higher the maximum permissible value for dynamic quenching of 2.13 x 10^{10} L. mol^{-1} s^{-1}. This clearly suggests that the quenching is static rather dynamic mode of quenching from the lifetime measurement.

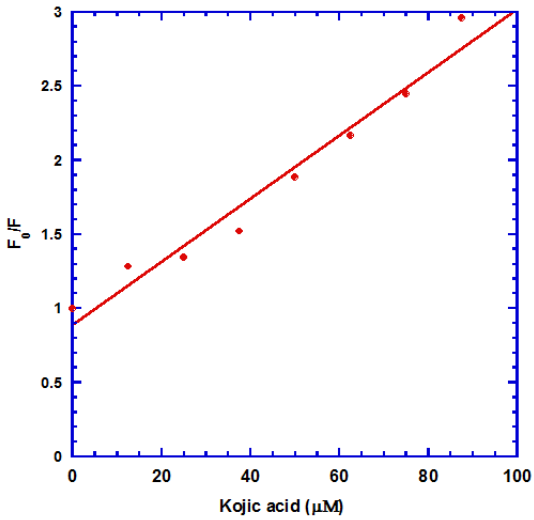

Figure 2: Stern-Volmer's plot of (F_0/F vs [Kojic acid]) for the quenching of β-amylase by Kojic acid at 298 K temperature and pH 7.4.

Binding constant and number of binding sites

Quenching of intrinsic fluorescence by various ligands is often used to assess the binding parameters (Eftink and Ghiron, 1976). The binding constant, Ka, and the number of binding sites, n, of kojic acid for β-amylase, were calculated using the Scatchard equation (equation 3). The linear regression plot of the interaction between β-amylase and Kojic acid at 25°C is shown in (Fig. 4). The values of K_a and n were obtained from the slope and the intercept, respectively. Ka value reflects the ability of kolaflavanone binding to β-amylase. The value of n from the slope was 1.08. It revealed that one molecule of kojic acid can bind tightly to β-amylase. The binding constant, Ka, was 1.55 X 10^3 L mol^{-1} (Table 1). This was slightly higher than 1.0 X 10^3 L mol^{-1}, representing a moderately favourable and

comparatively moderate ligand-protein interaction. The expected value of non-specific organic ligands-protein interaction using fluorescence spectroscopic methods is between 10^3- 10^4 L.mol^{-1} (Bourassa *et al*, 2013).

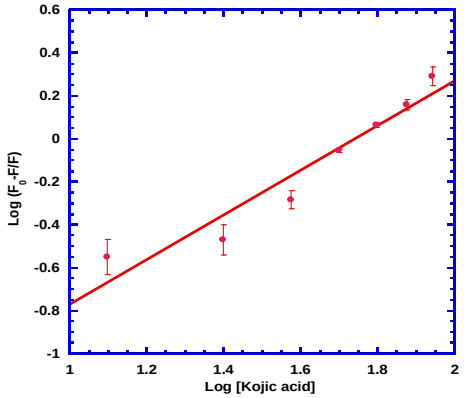

Figure 3: Scatchard Plot (Log (F_0-F)/F versus Log [Kojic acid]) for the binding of Kojic acid to β-amylase.

Table 1: The Stern Volmer quenching constant (Ksv) and association constant (Ka) of the interaction of Kojic acid with β-amylase at 298 K (pH 7.4).

Complex	K_{sv} (L. mol $^{-1}$)	K_q (L. mol^{-1})	Number of binding sites (n)	K_a (L. mol $^{-1}$)
β-amylase-Kojic acid	2.13 X 10^2	2.13 x 10^{10}	1.08	1.55 X 10^3

CONCLUSION

In this study, the interaction between β-amylase and Kojic acid was studied by fluorescence spectroscopy under simulative physiological conditions. Fluorescence experiments results revealed that the intrinsic fluorescence of β-amylase was quenched through static quenching process. This work, having estimated the binding affinity of a potential drug to a functional protein, opens an appealing perspective to understanding the possible pharmacological and toxicological action of kojic acid as well as revealing a subtle nano-vehicle function of the β-amylase for the ligand.

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