

EFFECT OF IONIZING RADIATION ON THE ACTIVITY OF THE ENZYME INVERTASE STUDIED BY POLARIMETRY

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Abstract

The effect of ionizing radiation on biological systems, and particularly the enzymes which are responsible for most cellular functions, leads to chemical alterations in their molecular structure, and as a subsequent result often causes inhibition of their activities. Of particular importance for understanding the molecular and kinetic mechanisms of action of ionizing radiation on these systems is to find suitable experimental protocols and kinetic models applicable to specific enzymes and their substrates. In the present study we offer a convenient experimental approach by applying polarimetric measurements to study the influence of ionizing radiation on the catalytic action of the enzyme invertase. Following the Michaelis–Menten kinetic model we determined the values of the kinetic constants K_m и $V_{\max}$ for the reactions catalyzed either by the native or irradiated invertase and made some conclusions about possible changes either in the active centre or in the molecular structure of the enzyme under the action of ionizing radiation.

Key words: invertase, enzyme kinetics, ionizing radiation, polarimetry, Michaelis–Menten constant

Introduction. Of all the physical effects on living systems and organisms, ionizing radiation is of specific importance since it initiates alterations in most biological processes [1], and irradiating the populations with small doses has an

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eminent impact on ecosystems [2]. During the last half-century, the interaction of ionizing radiation with organic matter has been extensively studied and described in numerous publications [3]. The main aspects and emphasis of these studies have always been related to the indirect influence of ionizing radiation through formation of radicals that are products of radiolysis. These reactants trigger a cascade of reactions that chemically affect intracellular stoichiometry and generate additional cell-damaging molecules called radiomodifiers [4]. Because they are predominantly involved in the processes of activation and inactivation of proteins, e.g. enzyme systems, the mechanisms and kinetics of their action have provoked a growing research interest over the past century [5]. Radiation inactivation studies of enzymes *in vitro* give important information about the basic properties of enzyme molecules, e.g. the amino-acid composition of their active sites, conformation changes, etc., but most importantly, they provide knowledge about function, migration, and interaction of the free radicals within the protein macromolecules [6]. There are too many known enzymes where radiation-induced damage can have a profound effect on cellular systems, but the properties of a few exposed to ionizing radiation have been investigated [6], e.g., mainly proteases have been the subject of investigation, mostly because these enzymes play a crucial role in the processing of proteins [7]. The invertase (β -fructofuranosidase) (E.C.3.2.1.26) is an enzyme that belongs to the group of glycosylated enzymes. It hydrolyzes the sucrose to glucose and fructose and has a relatively high activity that reaches a maximum at about 55 °C with pH optimum around pH 4.5 [8, 9]. It was reported that for the inactivation of yeast invertase by irradiation in a dilute aqueous solution, the increased rate of inactivation versus increasing dose is due to the contribution of radical species from radiolysis. It was also hypothesized that the destruction of methionine and histidine residues might be responsible for the free radical-induced inactivation of this enzyme [10]. The main aim of this study is to further explore the kinetic aspects of the inactivation of invertase undergoing the action of ionizing radiation. Accordingly, we proposed a new experimental approach employing polarimetric measurements to quantitatively assess ionizing radiation's influence on the catalytic action of invertase and find out how it changes the values for the kinetic constants K_m and V_{max} .

Materials and methods. Invertase extracted from baker's yeast (*Saccharomyces cerevisiae*) – 32 units/mg, was dissolved in acetate buffer (pH 4.7) to a stock solution with 1.35 mg/ml concentration and was further diluted with standard acetate buffer pH 4.7 to a concentration of 0.135 mg/ml. This enzyme solution was distributed in plastic 5.5 ml test tubes which were subsequently irradiated in a controlled manner at room temperature using a Cobalt-60 source at 160 mGy/min, so that ultimately, together with non-irradiated (control) samples, we had five more enzyme samples irradiated at temperature 23.8 °C and pressure 952 hPa with absorbed doses: 0.106 ± 0.005 Gy; 0.496 ± 0.025 Gy; 0.988 ± 0.05 Gy; 4.9 ± 0.2 Gy, and 9.78 ± 0.5 Gy, respectively. Doses were measured with a reference

ionization chamber irradiated together with the enzyme solution samples. For the experiments, the irradiated enzyme samples were diluted and used with a final concentration of 0.045 mg/ml. Sucrose (Valerus Ltd., Bulgaria) with 99.5% purity was dissolved in acetate buffer (pH 4.7) to 1 M stock solution which was further diluted to five final sucrose concentrations of 0.2 M, 0.1 M, 0.075 M, 0.05 M, and 0.025 M. Double-distilled water was used for all solutions and the ambient temperature of $T = 23^{\circ}\text{C}$ was maintained during all procedures.

For the measurements of optical angles of rotation of the polarized light, the disc polarimeter (LEOI-110, Lambda Scientific, USA) provided an accuracy $\pm 0.05^{\circ}$. First, for different sucrose concentrations spanning from 0.025 M to 0.25 M we obtained the calibration curve that gives the linear relationship between the sucrose concentration versus the optical angle of rotation α_c , i.e. $C_{\text{sucrosa}} = a\alpha_c$. The “coefficient of conversion” a converts the measured angles into respective molar concentrations of sucrose in mol/l and is found to be $a = 0.05$. Next, the invertase was added to a sucrose solution to the final enzyme concentration of 0.135 g/l. After that the reaction mixture was transferred into the polarimeter’s tubule and the changes in the angle of rotation were recorded at time intervals of 1 min. Each measurement was repeated three times and using the “coefficient of conversion” the obtained data were graphically represented as $C_t = C_t(t)$. To determine the initial rate of the reaction, only the points up to the tenth minute were collected and the best-fit line expressed by $C_t = -v_0t + C_0$, was obtained by the method of least squares. The slope of the curve v_0 represents the initial rate of the enzyme reaction. Further, the kinetic data for each irradiated solution were measured and fitted linearly so that the initial rates v_0 for each irradiated invertase solution were derived.

Results and discussion. The rate of almost any enzyme reaction varies over time, as a result of the influence of some factors, such as a decrease in the substrate concentration, inhibition of the enzyme by the product of the reaction, etc. Since the effect of these factors at the beginning of the reaction is insignificant, therefore it is generally accepted that the initial is determined by measuring the change of either product or substrate concentration only in the initial few minutes of the reaction. Following that rule, we found the initial rates v_0 for all irradiated invertases and graphed them versus the irradiation doses in Fig. 1A. The graph readily shows that the dependence of the enzyme activity, which correlates with the initial rate, decreases nonlinearly vs. the irradiation dose. Over the years various kinetic models have been developed and proposed to explain the trend of the enzyme activity as a function of the absorbed dose [11]. Most of them describe the tendency of the dilute enzyme solutions to decrease their activity when irradiated with increasing radiation doses, because “deactivated” enzymes compete with the active ones for the products of water radiolysis. In most of these studies it was found that the dependence of the enzyme activity on the radiation dose is linear, and the deviations from the experimental data were

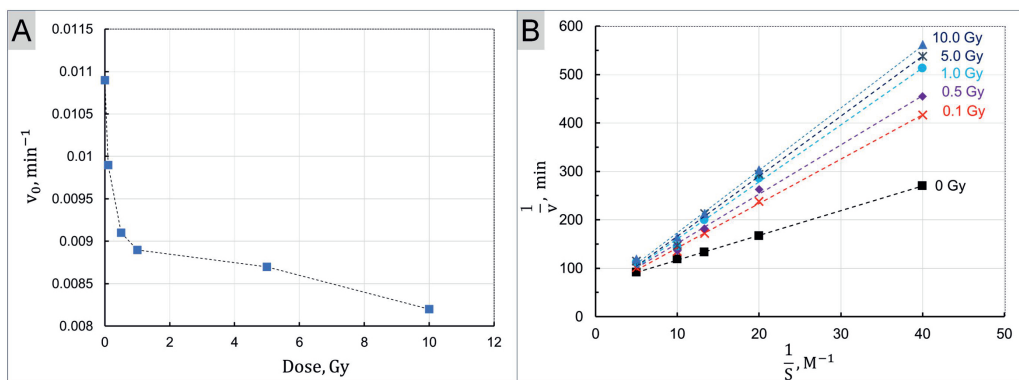


Fig. 1. (A) Dependence of the initial rates of the enzyme-catalytic reaction on the radiation dose for the enzyme invertase. (B) Graphically represented Lineweaver–Burk linearization $\frac{1}{v} = f\left(\frac{1}{[S]}\right)$ for the three concentrations of sucrose at different invertase irradiation doses. The graphs show the experimentally measured values and the lines obtained by the method of least squares. The dotted lines connect the points on the graphs only to trace the trend of the data

most often ignored. Later, however, it was acknowledged that if inactivated and activated enzyme molecules interact with aqueous reagents (primarily free radicals) at approximately equal reaction rates then modified kinetic models predict an exponential relationship between the dose and enzyme deactivation [11]. To elucidate the mechanism of the action of ionizing radiation, it is necessary to conduct systematic studies with different concentrations of enzyme and substrate (in this case the sucrose) and to extract the kinetic constants of the reactions, e.g. the Michaelis–Menten constant. For the five final concentrations of sucrose substrate in this study – 0.2 M, 0.1 M, 0.075 M, 0.05 M, and 0.025 M, respectively, we obtained the kinetic curves $C_t = -v_0t + C_0$ for each of these substrate concentrations and from them derived the initial rates of the enzyme reactions catalyzed by each of the irradiated invertase solutions (data not shown). Next, we used this data, following the Michaelis–Menten kinetic model, to determine the constants K_m and $V_{\max}$. Although the hypothesis of steady state in enzyme-catalytic reactions, which follows the kinetic formalism of Michaelis–Menten according to the equation $v = V_{\max} \frac{[S]}{K_m + [S]}$, introduces some ambiguities related to the exact interpretation and mainly the experimental determination of K_m and $V_{\max}$ [12], still the generally accepted concepts remain, namely that the K_m constant can be considered the dissociation constant of the enzyme-substrate complex ES , while $V_{\max}$, is the maximum rate of the reaction that is reached at the substrate concentration at which all the enzymes are saturated with it. On the other hand, the ratio $\frac{V_{\max}}{K_m}$ proves to be especially useful for the kinetic analysis. If we assume that in the Michaelis–Menten equation, $[S] \ll K$, then $[S]$ in the denominator $\frac{[S]}{K_m + [S]}$ can be neglected and we will get the rate of the enzyme-catalytic reaction

$v = \frac{V_{\max}}{K_m} [S]$. Consequently $\frac{V_{\max}}{K_m}$ can not only be considered a rate constant of a first-order reaction, but more specifically $\frac{V_{\max}}{K_m}$ is the rate constant of the reversible formation of a “productive” enzyme-substrate complex, i.e. $\frac{V_{\max}}{K_m}$ would give us a measure of the rate at which the substrate is “captured” in the active site of the enzyme and forms “productive complexes” which will further, at the corresponding rate, deliver the reaction product and “release” the enzyme from it. Hence this interpretation in many cases allows $\frac{V_{\max}}{K_m}$ to be interpreted as an equivalent to the catalytic constant k_{cat} , that is the turnover number for each enzyme reaction.

Figure 1B presents the Lineweaver–Burk linearization of the experimental data that was further used for estimation of K_m and $\frac{V_{\max}}{K_m}$. The graphs give the dependence of the reciprocal of the initial rates versus the reciprocal sucrose concentration, i.e. $\frac{1}{v} = f\left(\frac{1}{[S]}\right)$. The line slopes give a clear tendency of increasing effect of ionizing radiation when the radiation dose increases. The smallest slope of the curve that corresponds to the non-irradiated enzyme sample, as expected predicts the lowest value for K_m , which in turn corresponds to the greatest affinity of the enzyme to the substrate. As the radiation dose increases the K_m also increases, hence the ability of the substrate to dissociate from the enzyme also increases. Comparing the Michaelis–Menten constants of non-irradiated and irradiated invertases it is found that the Michaelis–Menten constant of the non-irradiated enzyme ($K_m = 76.5$ mM) is almost 2.5 times bigger than K_m reported elsewhere, which though, was obtained at the optimal for the invertase activity temperature of 55 °C [13]. On the other hand, the K_m of the non-irradiated enzyme is almost twice as small compared to the K_m of the immobilized invertase which had $K_m = 139.2$ mM and is comparable to K_m for the free invertase ($K_m = 93.2$ mM) [14].

Furthermore, compared to the constants K_m , obtained for irradiated invertases in our experiments at doses from 0.1 to 1 Gy (Fig. 2, triangles) one can estimate that their values which varied between $K_m = 173$ mM and $K_m = 300$ mM are almost 2 to 4 times bigger compared to the values of K_m of non-irradiated invertase. These data for K_m imply that the ionizing radiation most likely causes changes related to some chemical or structural modifications of the active site of the enzyme, thus acting on the catalytic activity in a way similar to the competitive invertase inhibitors [13]. To underline the observed trend that as K_m increases, the constant $\frac{V_{\max}}{K_m}$, which is directly related to the enzyme activity, decreases, the values of $\frac{V_{\max}}{K_m}$ are plotted versus the irradiation dose together with the data for K_m in Fig. 2. In most studies the data for the rate of enzymes’ inactivation versus increasing radiation dose fit a straight line when the remaining enzyme activity is plotted versus the radiation dose in a semilogarithmic plot [15]. Assuming that the inactivated enzyme molecules compete with the active ones for the agents formed from water, some theoretical equations predict an exponential dose-inactivation relationship [16]. If we analyze the related to enzyme activity

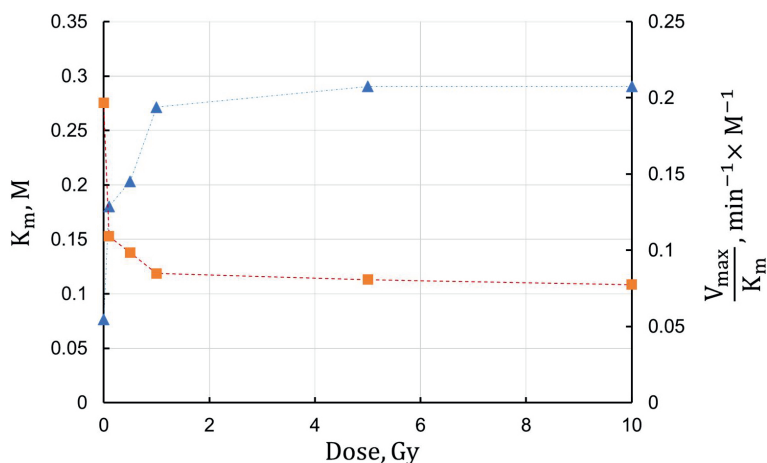


Fig. 2. The values of K_m (▲) and $\frac{V_{\max}}{K_m}$ (■) are plotted versus the irradiation dose. Dotted lines connect the points on the graph only to trace the trend of the data with increasing the radiation dose

values of v_0 (Fig. 1A) and $\frac{V_{\max}}{K_m}$ (Fig. 2, squares) versus the increasing radiation dose, then we can conclude that if the dose is increased up to 10 Gy, then v_0 and $\frac{V_{\max}}{K_m}$ decrease by 75% and 37.5%, respectively. Therefore, to make any conclusions about the relationship between enzyme activity vs. dose we need data for higher doses of irradiation.

Conclusions. In this article, the influence of different doses of ionizing radiation on the catalytic action of the enzyme invertase was investigated by means of polarimetric measurements. By following such an experimental approach, we obtained kinetic curves for the rate of the enzymatic reaction of sugar inversion of sucrose with either native or irradiated at different doses enzyme solutions of invertase. We also found the exact values of the kinetic constants K_m and $V_{\max}/K_m$. The data show that the values of K_m for irradiated enzymes were about four times bigger than the value of K_m for the non-irradiated enzymes, which implies that the radiation most likely led to some chemical modifications of the enzyme active site and has acted similarly as the competitive inhibitors of the invertase [13].

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