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INHIBITION OF PLANT GROWTH REGULATORS ON PARAOXONASE ACTIVITY

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ABSTRACT

The purpose of this study was to investigate the *in vitro* effects of four commonly used plant growth regulators (PGRs) (indole-3-acetic acid, indole-3-butyric acid, gibberellic acid and kinetin) on human serum paraoxonase (PON). Serum paraoxonase is an esterase that plays a key role in organophosphate detoxification and in prevention of atherosclerosis. Serum paraoxonase from humans was purified using ammonium sulfate precipitation and Sepharose-4B-L-tyrosine-1-napthylamine hydrophobic gel. IC_{50} values of the chemicals that cause inhibition were determined by means of activity percentage diagrams. The plant growth regulators used in this study affected the PON activity from human serum to various degrees. The concentrations of in-dole-3-acetic acid, indole-3-butyric acid and gibberellic acid that inhibited 50% of the enzymatic activity were 63.86µM, 45.48µM and 14.4mM, respectively. Conversely, the enzyme activity was increased by kinetin.

KEYWORDS: Paraoxonase, indole-3-acetic acid, indole-3-butyric acid, gibberellic acid, kinetin, purification, inhibition.

INTRODUCTION

Paraoxonase 1 (PON1 EC 3.1.8.1) is a member of a family of enzymes (PON1, PON2 and PON3) that are widely spread in mammals, such as rats, rabbits, and mice, as well as humans, but are also found in many other species [1] The two PON1 isoenzymes Q and R are by far the most investigated family members. They are calcium-dependent hydrolases that catalyze the hydrolysis of a broad range of esters and lactones [2] PON1 is also catalyzes, albeit at much lower rates, the hydrolysis and thereby inactivation of various organophosphates, including the nerve agents sarin and soman [3] Initial interest in PONs was therefore, toxicological. Two substrates, paraoxon and phenyl acetate, have been used in the routine assay of PON1 activity, because the same active site of PON1 is responsible for the hydrolysis of both substrates [4]

In addition, PON1 is involved in drug metabolism and is being used for drug inactivation [2, 5] In recent years, it has become apparent that PONs play an important role in the prevention of atherosclerosis. PON1 resides in the cholesterol-carrying particles high-density lipoprotein (HDL, ‘‘good’’ cholesterol). The levels of PON1 in the blood and its catalytic proficiency appear to have a major impact on the individual’s susceptibility to pollutant and insecticide poisoning and to atherosclerosis [6] Furthermore, mice lacking the PON1 gene are highly susceptible to atherosclerosis and to OP poisoning [7] Given the physiological importance of the paraoxonase, the metabolic impact of chemicals used for crop production should receive greater study. However, there is not much inhibition study available on paraoxonase activity.

Many chemicals are currently used in agriculture, and PGRs are among those widely used. The amount and variety of PGRs which include indole-3-acetic acid, indole-3-butyric acid, gibberellic acid, and kinetin have increased

tremendously in recent years. Indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) are naturally occurring plant growth regulators extensively studied by phytochemists (Fig 1a, b). IAA and IBA are major plant growth hormones of the auxin class, affecting cell enlargement, division, and differentiation [8] It is metabolized by two different pathways conjugation with a variety of amino acids, peptides, and sugars forming non-reactive conjugates and oxidation by peroxidases leading to production of a toxic series of intermediates which could be used as the basis of a novel cancer therapy [9-11] Gibberellic acid (GA_3) (Fig 1c) plays important roles in many cellular processes including promotion of stem elongation overcoming dormancy in seed and buds involvement in parthenocarpic fruit development, flowering, mobilization of food reserves in grass seed germination, juvenility, and sex expression [12] The amount of these substances placed into the environment may soon exceed those of insecticides [13] Kinetin (Kn) (Fig 1d) was isolated 50 years ago for the first time as a plant hormone. So this hormone has been used for a long time [14]

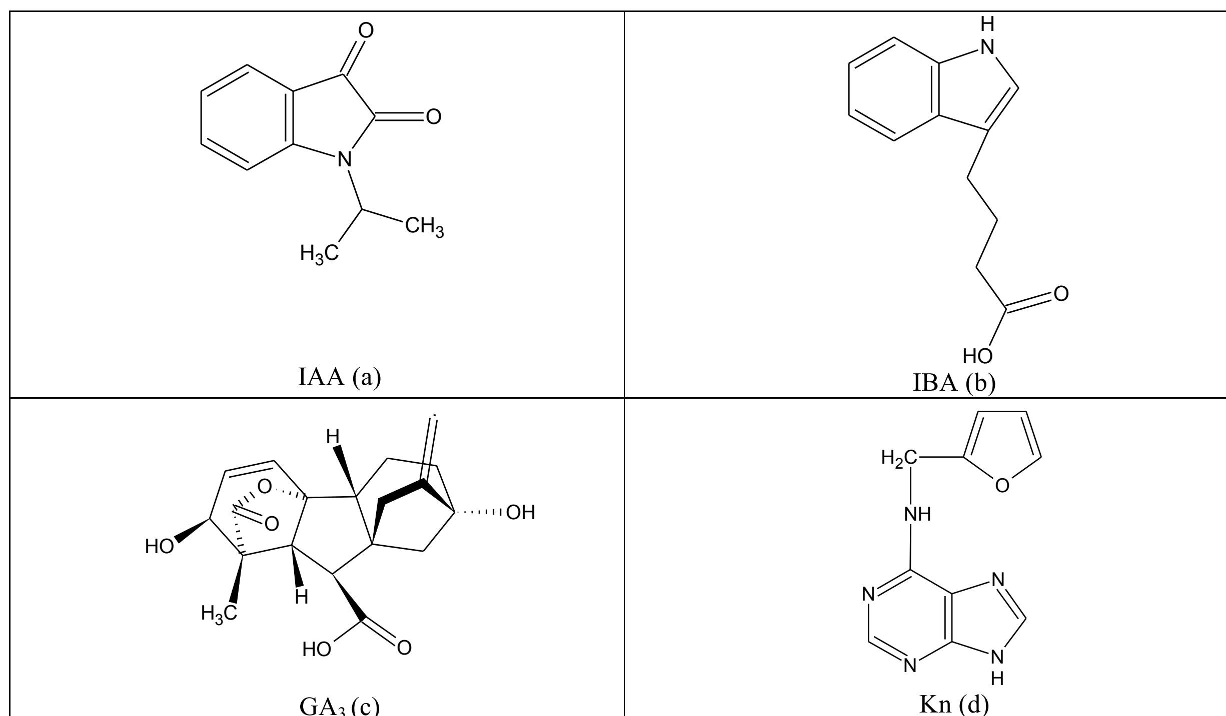

FIGURE 1 - Structure of (a) indole-3-acetic acid (IAA), (b) indole-3-butyric acid IBA, (c) gibberellic acid (GA_3), and (d) kinetin (Kn)

In the literature, it is reported that IAA might induce the neuronal apoptosis in the S phase and lead to microencephaly [15] Also, de Melo et al. [16] determined that incubation for 24 h in the presence of IAA (1 mM) showed increase in the activities of SOD, CAT, and glutathione peroxidase. John et al. [17] observed that IAA possesses teratogenic effects in mice and rats. In addition, it was found that gibberellin A_3 induced liver neoplasm in Egyptian toads, and they suggested that the tumors could be diagnosed as hepatocellular carcinomas [18] Ozmen et al. [19] observed that abscisic acid and gibberellic acid affect on sexual differentiation and some physiological parameters of laboratory mice. The effects of IAA and Kn were also investigated on human serum enzymes in vitro. IAA was found to inhibit aspartate aminotransferase and activate amylase, creatine phosphokinase and lactate dehydrogenase. Kn inhibited muscle creatine kinase while it activated aspartate aminotransferase and alanine aminotransferase [20] Also, it was found that while the levels of LDH and CPK were increased significantly by IBA (indole butiric acid), the levels of AST, LDH, and CPK were increased significantly by IAA. In addition, the levels of AST, LDH, and CPK were increased significantly by kinetin [21] Hsiao's [22] results suggest that kinetin has effective free radical-scavenging activity in vitro and antithrombotic activity in vivo. On the other hand, some PGRs have been shown to affect the carbonic anhydrase isoenzymes of erythrocytes in humans and bovines [23]

Although PGRs are used for pest control and increased productivity on a wide variety of crops, little is known about the biochemical or physiological effects in mammalian organisms. Therefore, in this study in vitro inhibition of some important PGRs (indole-3-acetic acid, indole-3-butyric acid, gibberellic acid, kinetin) on serum PON1 was evaluated in humans.

MATERIALS AND METHODS

Materials

Sepharose 4B, L-tyrosine, 1-napthylamine, protein assay reagents and chemicals for electrophoresis were obtained from Sigma Chem. Co. All other chemicals used were of analytical grade and obtained from either Sigma or Merck. Plant growth regulators were provided by the local pharmacy.

Paraoxonase Enzyme Assay

Paraoxonase enzyme activity towards paraoxon was quantified spectrophotometrically using the method described by Gan *et al.* [24] The reaction was monitored for 2 min at 37 °C by monitoring the appearance of the *p*-nitrophenol line at 412 μm in a Biotek automated recording spectrophotometer. The assay buffer contained 0.1 M Tris–HCl (pH 8.5), 2 mM $CaCl_2$, 50 μL purified enzyme and paraoxon. The final substrate concentration during enzyme assay was 2mM, and all rates were measured in two separate trials and corrected for the non-enzymatic hydrolysis.

Total Protein Determination

Absorbance at 280 nm was used to monitor the protein in the column effluents and ammonium sulfate pre-

cipitation. Quantitative protein determination was achieved by absorbance measurements at 595 nm according to Bradford [25] with bovine serum albumin as a standard.

Purification of Paraoxonase from Human Serum by Hydrophobic Interaction Chromatography

Human serum was isolated from 35 ml of fresh human blood and put into a dry tube. The blood samples were centrifuged at 1500 rpm for 15 min and the serum was removed. Then serum paraoxonase was isolated by ammonium sulfate precipitation (60-80%). The precipitate was collected by centrifugation at 15000 rpm for 20 min, and redissolved in 100mM Tris–HCl buffer (pH 8.0). Next, we synthesized the hydrophobic gel, including Sepharose 4B, L-tyrosine and 1-napthylamine, for the purification of human serum paraoxonase [27] The column was equilibrated with 0.1 M of a Na_2HPO_4 buffer (pH 8.0) including 1 M ammonium sulfate and 3ml enzyme solution was loaded. The paraoxonase was eluted with an ammonium sulfate decrease gradient using 0.1 M Na_2HPO_4 buffer with and without ammonium sulfate (pH 8.0). The purity of the enzymes was confirmed with SDS gel electrophoresis (Figure 2). The purified PON1 enzyme was stored in the presence of 2mM calcium chloride in order to maintain activity.

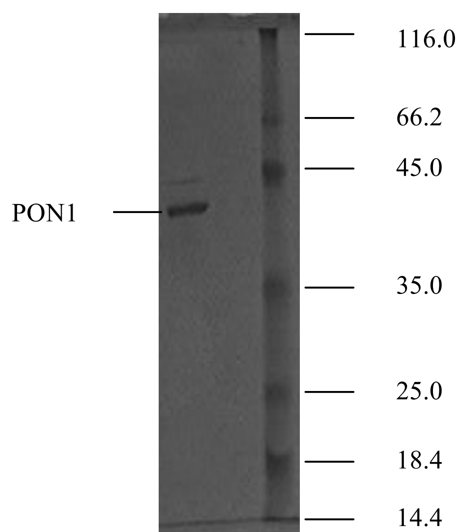

FIGURE 2 - SDS-PAGE of Saanen goat carbonic anhydrase. The poled fractions from hydrophobic interaction chromatography (Sepharose 4-B, L-tyrosine, 1-Napthylamine) was analyzed by SDS-PAGE (%12 and %3) and revealed by Coomassie Blue staining. Experimental conditions were as described in the methods section. Lane 1 contained 5 µl of various molecular mass standards: β-galactosidase, (116.0), bovine serum albumin (66.2), ovalbumin (45.0), lactate dehydrogenase, (35.0), Restriction endonuclease (25.0), β-lactoglobulin (18.4), lysozyme (14.4).

SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was performed in order to verify the purified enzyme. It was carried out in 12% and 3% acrylamide concentrations for running and stacking gel respectively, containing 0.1% SDS according to following Laemelli *et al.* [26]

In Vitro Inhibition Kinetic Studies

For the inhibition studies of indole-3-acetic acid, indole-3-butyric acid, gibberellic acid and kinetin, different concentrations of PGRs were added to the enzyme activity. Paraoxonase activity with PGRs was assayed by following the hydration of paraoxon. Activity % values of paraoxonase for five different concentrations of each PGRs were determined by regression analysis using Microsoft Excel. Paraoxonase activity without a PGRs was accepted as 100% activity. For PGRs having an inhibition effect, the inhibitor concentration causing up to 50% inhibition (IC_{50} values) was determined from the graphs.

RESULTS AND DISCUSSION

Inhibition graphs, using the PGRs with concentrations as described in Section 2.5 are shown in Figure 3. Different inhibition effects of the applied PGRs were obtained. IC_{50} values of the enzyme inhibited by indole-3-acetic acid, indole-3-butyric acid, and gibberellic acid were found to be 63.86µM, 45.48µM and 14.4mM, respectively. The auxin hormone indole-3-acetic acid has been shown to be the strongest inhibitor against the PON1 activity (Figure 3-a). Conversely, kinetin considerably stimulated the enzyme activity at the applied concentrations (Figure 3c).

The amount and variety of PGRs used has increased tremendously in recent years. This increase has caused a positive effect on crop production, however, certain regulators, their residues, metabolites and/or contaminants have created many unforeseen adverse effects on the environment. Under some conditions, PGRs may be present in very low concentrations which have no immediate detectable effect. These small amounts of chemicals can cause sublethal damage to organisms and this is more insidious and difficult to define than acute toxicity. Sublethal effects may be further enhanced by persistent PGRs which are accumulated in the organisms and magnified in the food chain. In this study, IAA, IBA, GA_3, and Kn were preferred because there is no information about their side effects on serum PON1. PGRs which were used in this study are found in plants as endogen hormones and wide variety of biologically active compounds.

We found that activity of human serum PON1 was inhibited by IAA, IBA, and GA_3. Conversely, Kn was stimulated the activity of serum PON1. Many other reports support our study. For example, it is reported that a serum enzyme named aspartate aminotransferase, amilase, creatin phosphakinase, and lactate dehydrogenase was inhibited by IAA [20] Similar *in vitro* data found that glutathion peroxidase and catalase was strongly inhibited by IAA and kinetin [28] In addition, *in vivo* reports showed that, IAA has inhibition effects on glutathion reductase and glutathion peroxidase [29] This enzyme has an important role in antioxidant defense system as PON1. The decreased activity of PON1 may lead to decreased protection against lipid oxidation [30]. The increase of lipid oxidation directly effects on to take a risk existence of atherosclerosis and organophos-

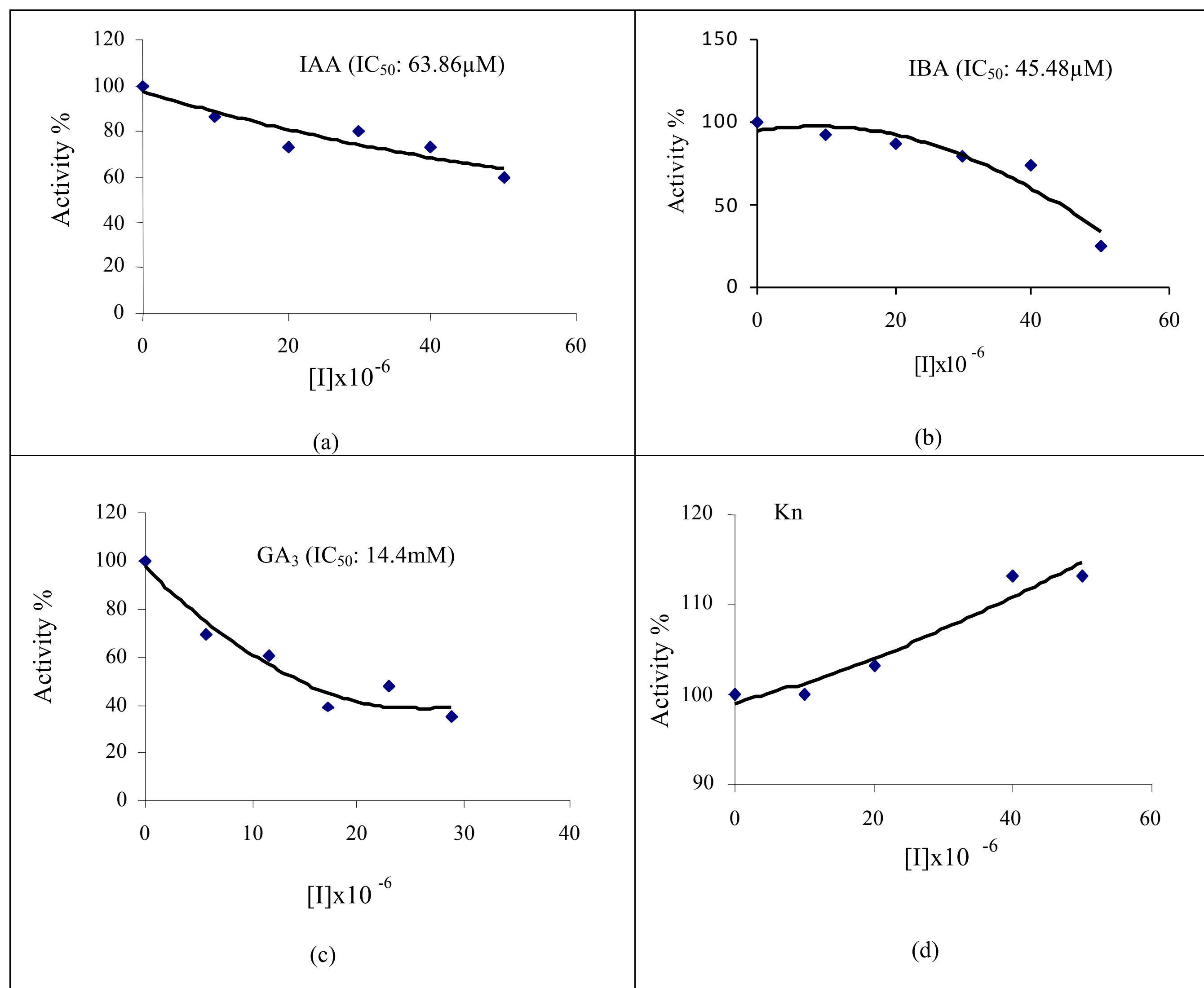

FIGURE 3 - Activity (%) curves of PON1 for different concentrations of IAA,IBA, GA_3, Kn.

phate sensitivity. IAA and IBA are major plant growth hormones of the auxin class, affecting cell enlargement, division, and differentiation. Although this regulator is indispensable for plant growth, it showed inhibition effects on PON1 which has an important physiological role of organism. Also GA_3 does not inhibit PON1 activity as much as other regulators. It is reported that GA_3 significantly decreased serum aspartate aminotransferase, creatine phosphokinase, and lactate dehydrogenase. Antioxidant enzyme activities such as superoxide dismutase significantly decreased in the erythrocyte, liver and brain tissue of rats with GA_3 [31]

Kn is the first investigated and best known regulator. We determined that human serum PON1 activity was stimulated by kinetin. Kn is an anti-oxidant both *in vitro* and *in vivo*. It protects DNA against oxidative damage to 8-oxo-dG mediated by the Fenton reaction [32] Also Kn acts as a strong inhibitor of oxidative and glycoxidative protein-damage generated *in vitro* [14] However, another study showed that, exposure to Kn can result in toxicological effects in vertebrates. It was found that glutathion-S-transferase and catalase were inhibited by Kn in vivo [28] In another study showed that gltathion reductase, glutathion peroxidase, and adenosine deaminase were significantly reduced with Kn [29]

In conclusion, the aim of this study was to define the effects of these plant hormones on human serum paraoxonase and thus evaluate the environmental and toxicological effects of these compounds in vitro. Although, PGRs are a major component of the growing process in plants and cause a positive effect on crop production, they dramatically inhibit the human serum PON1. This finding is important, because PON1 is one of the most important enzymes which has antioxidant and antitoxicological ef-

fects in organisms. Even though they are consumed in small amounts, these three plant growth regulators can still affect PON1. Also, because they are magnified in the food chain the inappropriate use of PGRs presents a potential a risk to human health.

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