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PLANT PROTECTION

Initial Characterization of Glutathione S-Transferase from Larval Midgut of *Spodoptera littoralis* and Its Inhibition by Spinosad and *Beauveria bassiana* Using O-Dinitrobenzene as a Substrate

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Abstract. Initial characterization of glutathione S-transferase from larval midgut of *Spodoptera littoralis* and its inhibition by spinosad and *Beauveria bassiana* using O-dinitrobenzene as a substrate were studied. The results showed: (i) the glutathione S-transferase (GST) from larval midgut of *S. littoralis* is similar in its properties to those that have been studied in vertebrate organisms;

(ii) activity of GST was inhibited by spinosad and *B. bassiana* with I_{50} values of 15.06 nM (49.69 ppm) and 53.26 ppm respectively;

(iii) the kinetic study of GST showed that the k_m and v_{max}. values of *O*-dinitrobenzene (DNB) were 0.46 mM and 53.48 n mole/min, while the k_m and v_{max}. values of glutathione (GSH) were 1.43 mM and 87.72 n mole/min;

(iv) the inhibition constant (k_i) for the spinosad and *B. bassiana* were 60 nM (200 ppm) and 27 ppm respectively, therefor, *B. bassiana* is more potent effective on GST activity than spinosad; and

(v) the inhibition pattern of spinosad was a noncompetitive, whereas the inhibition pattern of *B. bassiana* was an uncompetitive.

Introduction

Extensive studies on insect enzymes involved the metabolism of xenobiotics have been done during the past several decades because these enzymes are essential in the metabolism of insecticides in insects. Glutathione S-transferase (EC.2.5.1.18) are a group of detoxification enzymes that act by catalyzing the conjugation of toxic compounds, by virtue of their possession of an electrophilic reactive center, with the nucleophilic thiol group of GSH. They are extremely widespread in nature [1,p 213-223], but detailed studies on the properties of these enzymes have to date been confined almost entirely to those isolated from mammals. Observations that resistance to insecticides in insects may be attributable to enhanced GSH-dependent detoxification [2,3] have focused interest on these enzymes in insect pest strains. In view of the role of glutathione S-transferase in protecting the organism from toxic injury [4,5], it is important to know what

environmental compounds alter their activity. Various methods have been proposed to assay the enzyme. Among these, the methods using 1-chloro-2,4-dinitrobenzene [6] and 1,2-dichloro-4-nitrobenzene [7] have been used most widely because of their high sensitivities. These substrates, however, show high blank values, and undergo rapid non-enzymatic reactions. For these reasons, measuring the initial reaction rate in a recording spectrophotometer usually performs the above assay, and thus it is difficult to assay many samples simultaneously. Therefor we are chosen *O*-dinitrobenzene as a substrate, the method based on the colorimetric determination of the liberated nitrite after diazo-coupling with N-(1-naphthyl)-ethylenediamine dihydrochloride and sulfanilamide. After termination of the reaction, the absorbance of the mixture was stable for 6 h at room temperature [8].

Spinosad (Tracer, DowElanco, Indianapolis, IN), a combination of spinosyns A and D which represents a naturally derived group of insect control molecules (known as the naturalytes) derived from a new species of actinomycete bacteria *Saccharoplyspora spinosa* [9]. Although spinosad provides a good contact activity, it is most toxic when ingested [10]. *Beauveria bassiana* natural fungal organism to control a wide range of insects including silver leaf / sweet potato whitefly, thrips, scales, aphids, and mites. Naturolis-L (Strain JW-1) for use on raw agricultural commodities and cotton [11]. Currently, there is a tremendous interest in the toxicity of the biopesticides spinosad and *B.bassiana* extract, partly because of their extensive use in the agriculture.

The present work aimed to test these biopesticides on the activity of glutathione S-transferase. Also, the aim has been to obtain information that will enable a detailed comparison of the properties of the enzymes from an insect with those of the mammalian enzymes that have been studied in depth. Such comparisons are necessary if it is ever to be possible to exploit species differences in the nature of GSH-dependent detoxification in the production of pesticides of high species-specificity.

Materials and Methods

Insect

Larvae of cotton leaf worm, *Spodoptera littoralis* (Lepidoptera:Noctuidae) were obtained from the Department of Plant Protection Insectry, Faculty of Agriculture, King Saud University. These larvae were reared in the laboratory on castor oil leaves for at least three years according to Eldefrawi *et. al.*[12]. Midguts of fifth instar larvae were used for enzyme assay.

Biopesticides

Spinosad is a mixture of spinosyn A and D, Molecular Weight 1477.98 (insecticides suspension concentrate 44.2% DE-105; Dow Elanco, Indianapolis, IN) and Biofly (*Beauveria bassiana*) 100% (w/w) from Egyptian strain of *B. bassiana* as a

suspension having at least $30x10^6$ conidia/ml, produce by El-Naser for fertilizers and biopesticides were used in enzyme assay as aqueous solution.

Chemicals

Reduced glutathione (GSH >99% purity) was obtained from Merck, Darmstadt. *O*-dinitrobenzene (DNB) was obtained from BDH chemicals, poole, United Kingdom. All the other chemicals were of the highest purity grade available from Sigma, BDH and or Aldrich chemical companies.

Enzyme source

Midguts of fifth larval instar (1 g approx.) were collected and homogenized in 10 ml of 0.1 M phosphate buffer pH 7 in Temkar homogenizer. The homogenate was centrifuged for 30 min at 15000 rpm at 4 °C using Beckman L5-75 ultracentrifuge type 40 rotor, and the supernatant served as enzyme source.

Standard assay for enzyme activity

Glutathione S-transferase activity was measured according the method of Asaoka and Takahashi [8] using *O*-dinitrobezene as a substrate with slightly modification. The standard assay mixture (1 ml) contained: 1.5 mM GSH, 0.1 M phosphate buffer, pH 7, 20 µl (30 µg protein) of enzyme solution and the reaction was started by the addition of 0.5 mM DNB dissolved in ethanol. After incubation at 37 °C for 20 min the reaction was terminated by the addition of 0.1 ml of acetic anhydride. The mixture was left for 5 min at room temperature, and then mixed with 1 ml of 1% (w/v) sulfanilamide in 20% (w/v) HCl followed by 1 ml of 0.02% (w/v) N-(1-naphthyl)ethylenediamine dihydrochloride. After diazo-coupling for 20 min at room temperature 0.1 ml of 1 % (w/v) ammonium sulfamate (freshly prepared) was added to the mixture. The mixture was centrifuged and left for 5 min, then the absorbance at 540 nm was measured in a uv-vis spectrophotometer (SHIMADZU UV-1201). An assay mixture without enzyme was used as the blank. Protein concentration was estimated by method of Lowry *et. al.*[13] using bovine serum as standard. Excel spreadsheet was used for calculation and graphs.

Results and Discussion

Effects of substrate concentrations on the assay

Figure 1 shows the activity of glutathione S-transferase determined at various concentrations of GSH and DNB under the standard conditions (reaction time, 20 min.). Figure 1a shows apparent GSH at 3 mM(but the absorbance over 1). On the other hand, no saturation was observed for DNB (Fig. 1b), but above 1 mM of DNB (10% ethanol in reaction mixture) slightly decrease in activity was observed. Based on these results, the concentrations of DNB and GSH were chosen to be 0.5 mM and 1.5 mM respectively, in the standard assay.

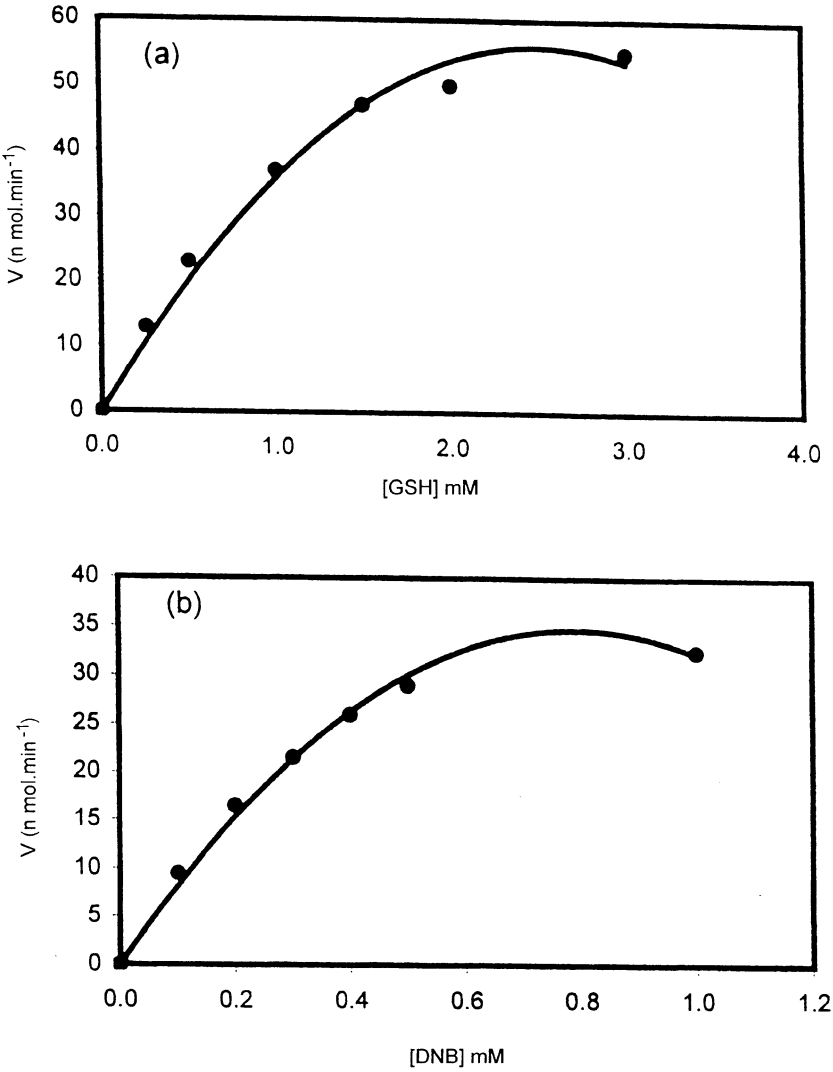

Fig. 1. Effects of substrate concentration on the assay. The enzyme extract (30 μg) was assayed under the standard conditions at pH 7 and incubation at 37°C for 20 min. except that various substrate concentrations were used (a) DNB, 0.5 mM; (b) GSH, 1.5 mM.

Linearity of enzyme reaction with respect to time

A linear relationship was obtained between the reaction time and the release of nitrite from the substrate up to 60 min with 30 μg of the crude enzyme (Fig. 2). All subsequent incubations in the present studies were performed for 20 min.

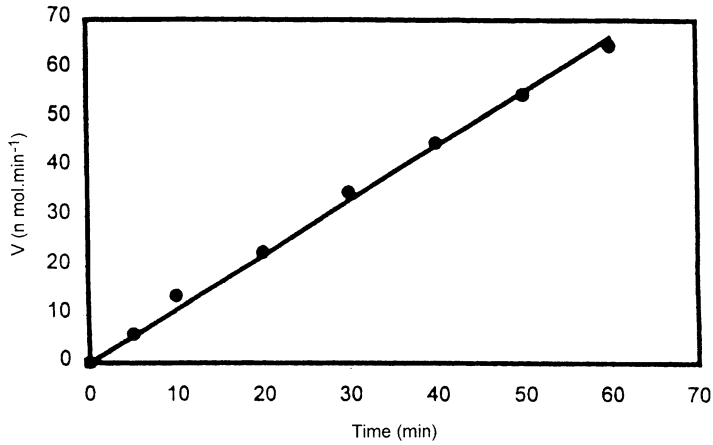

Fig. 2. Time course of the reaction with enzyme extract. Assay was performed for various periods under the standard conditions at pH 7 and 37°C, using 0.5 mM DNB and 1.5 mM GSH as substrates.

Proportionality of activity to enzyme concentration

Data in Figure 3 show typical results estimated by the standard assay method with a crude extract of enzyme obtained from larval midgut of *S. littoralis*. A linear relationship was obtained in the activity.

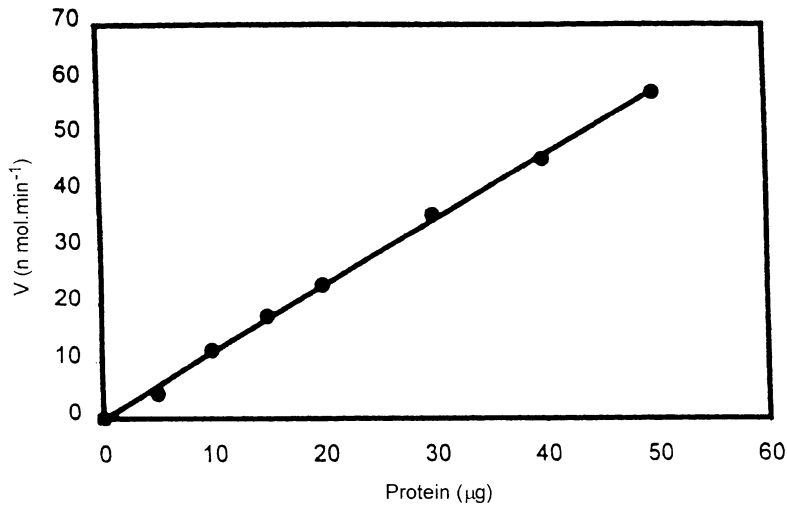

Fig. 3. Standard curve for assay of the enzyme extract. The activity of the enzyme extract of larvae midguts was determined at various protein concentrations by the standard assay method at pH 7 and incubation at 37°C for 20 min. using 0.5 mM DNB and 1.5 mM GSH as substrates.

Inhibitory action and kinetics studies of inhibited glutathion S-transferase by spinosad and *B. bassiana*

Glutathion S-transferase(GST) from larvae midguts of *S. littoralis* was titrated with increasing concentrations of spinosad and *B. bassiana* (biofly). Data in Fig. 4 show that GST was inhibited extensively by spinosad and *B.bassiana.*

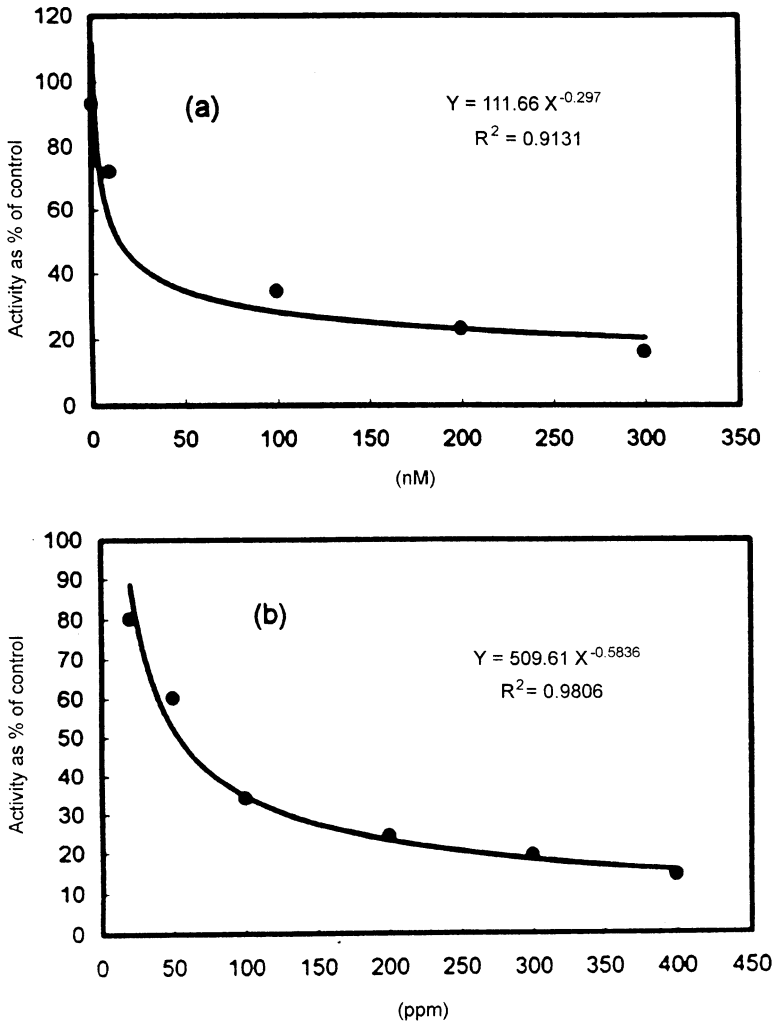

Fig. 4. Inhibition of glutathion S-transfersae by spinosad (a) and *B. bassiana* (b). Enzyme activity was measured by a standard assay in presence of the indicated concentrations of spinosad and *B. bassiana*.

The concentration of spinosad required for 50% inhibition (I_{50}) was 15.06 nM (49.69 ppm) [calculated by $Y = 111.66\ x^{-0.297}$, $R^2 = 0.9131$] (Fig. 4a), while I_{50} of *B. bassiana* was 53.26 ppm [calculated by $Y = 509.61\ x^{-0.5836}$, $R^2 = 0.9806$] (Fig. 4b). Sparks *et. al.*[14] suggested that while spinosyn A is initially slower to penetrate into lepidopterous larvae , once it is internalized, it is relatively stable to metabolism thereby contributing to its high level of activity. This means that, glutathione S-transferase in lepidopterous larvae may be a target to spinosad. Also, Schocken *et. al.* [15] reported that the filamentous fungus, *B.bassiana*, produced a methoxylated glycoside of the monohydroxylated metabolite with a yield of 80% dihydroxylated metabolites. Therefore the inhibition of GST by *B. bassiana* may be return to dihydroxylated metabolites. The obtained results are agree with those mentioned with Vessey and Zakin [16], they found that GST from rat liver is more sensitive to dihydroxy bile acids than trihydroxy bile acids.

The kinetic parameters of GST from larvae midguts of *S. littoralis* and their type of inhibition by spinosad and *B. bassiana* were studied as shown in Figs. 5-7. The k_m and $v_{max.}$ values of DNB were found to be 0.46 mM and 53.48 n mole/min [calculated by Y= 0.0086x + 0.0187, R^2= 0.9951] (Figs. 5a and 6a), while the k_m and $v_{max.}$ values of GSH were found to be 1.43 mM and 87.72 n mole/min [calculated by Y= 0.0163x + 0.0114, R^2= 0.9985] (Figs. 5b and 6b).

The inhibition of glutathione S-transferase by spinosad and *B. bassiana* were studied over a range of concentrations of the *O*-dinitrobenzene(DNB) and glutathione(GSH) at fixed concentrations of spinosad (Fig. 5) and *B. bassiana* (Fig. 6). In presence of spinosad the k_m value was unaffected ,whereas the $v_{max.}$ value was decrease when DNB and GSH respectively were the varied-concentration substrates (Figs. 5a and 5b). On the other, in presence of *B. bassiana* both the k_m and the $v_{max.}$ values were affected (Figs.6a and 6b) when DNB and GSH respectively were the varied-concentration substrates.

The inhibition was analyzed graphically by means of Dixon plots (Figs. 7a and 7b). Apparent inhibition constants (k_i) for the spinosad and *B.bassiana* were found to be approx. 60 nM (200 ppm) [calculated by Y= 0.0003x + 0.018, R^2=0.9995] and 27 ppm [calculated by Y= 0.0004x + 0.0108, R^2= 0.9999] respectively.

The previous results of double reciprocal plots (Figs. 5 and 6) and Dixon plots (Fig. 7) indicated that, the inhibition pattern of spinosad was a noncompetitive type, whereas the inhibition pattern of *B.bassiana* was an uncompetitive type.

The present results are in accordance with that reported for another insects glutathione S-transferase with 1-chloro-2,4-dinitrobenzene as substrate, from the larvae of *Galleria mellonella* and *Wiseana cervinata* in which the behavior of the enzyme with respect to the electrophilic substrate was Michaelian but was non-Michaelian with respect to GSH [17,18]. There are differences in detail between the kinetic patterns obtained with the *Wiseana* enzyme, with the rat liver enzyme and with the transferase isolated from *Galleria mellonella* [17]. For instance, whereas both insect enzymes exhibit product inhibition by NaCl, the rat liver transferase does not.

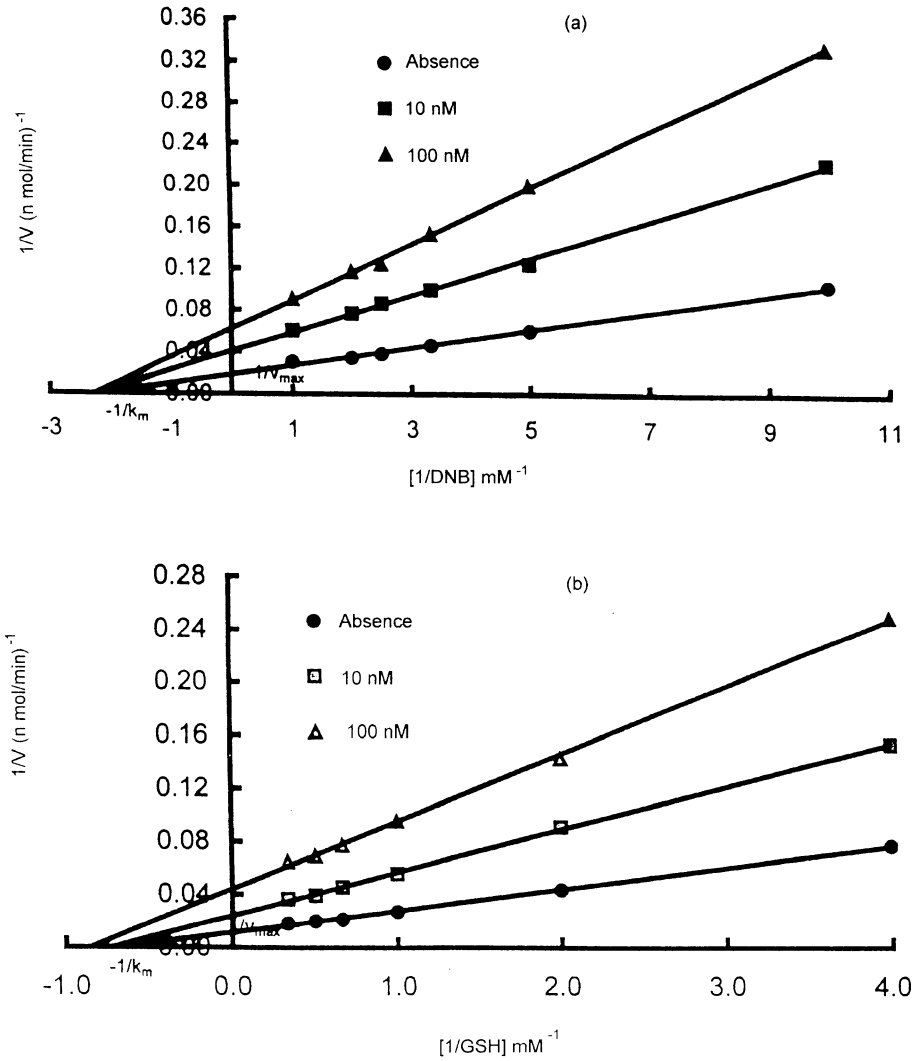

Fig. 5. Double reciprocal plots of GST activity as a function of the concentration of DNB (a) or GSH (b) in the presence or absence of spinosad. (a) Activity was determined in assay containing 1.5 mM of GSH and variable concentrations of DNB in presence or absence of spinosad. (b) The concentration of DNB was fixed 0.5 mM and the concentration of GSH was varied in presence or absence of spinosad.

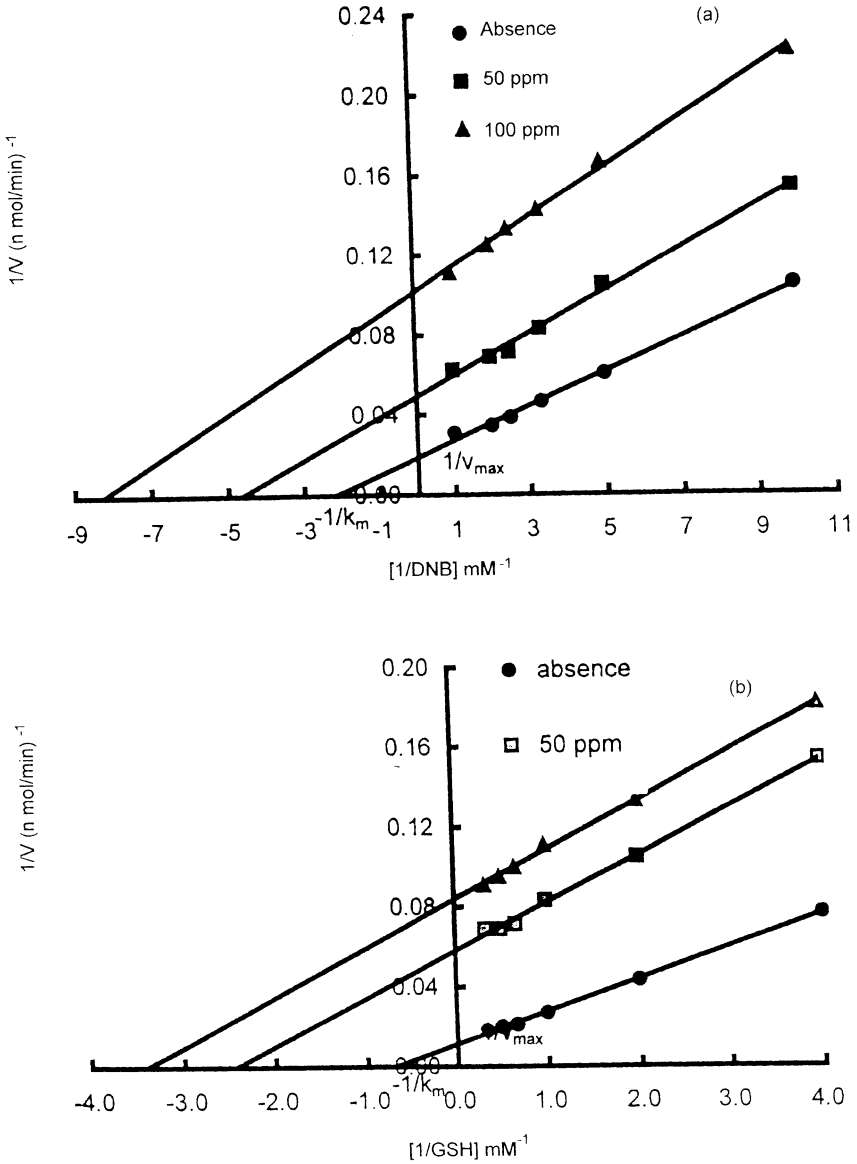

Fig. 6. Double reciproal plots of GST activity as a function of the concentration of DNB (a) or GSH (b) in the presence or absence of *B. bassiana*. (a) Activity was determined in assay containing 1.5 mM of GSH and variable concentrations of DNB in presence or absence of *B. bassiana*. (b) The concentration of DNB was fixed 0.5 mM and the concentration of GSH was varied in presence or absence of *B. bassiana*.

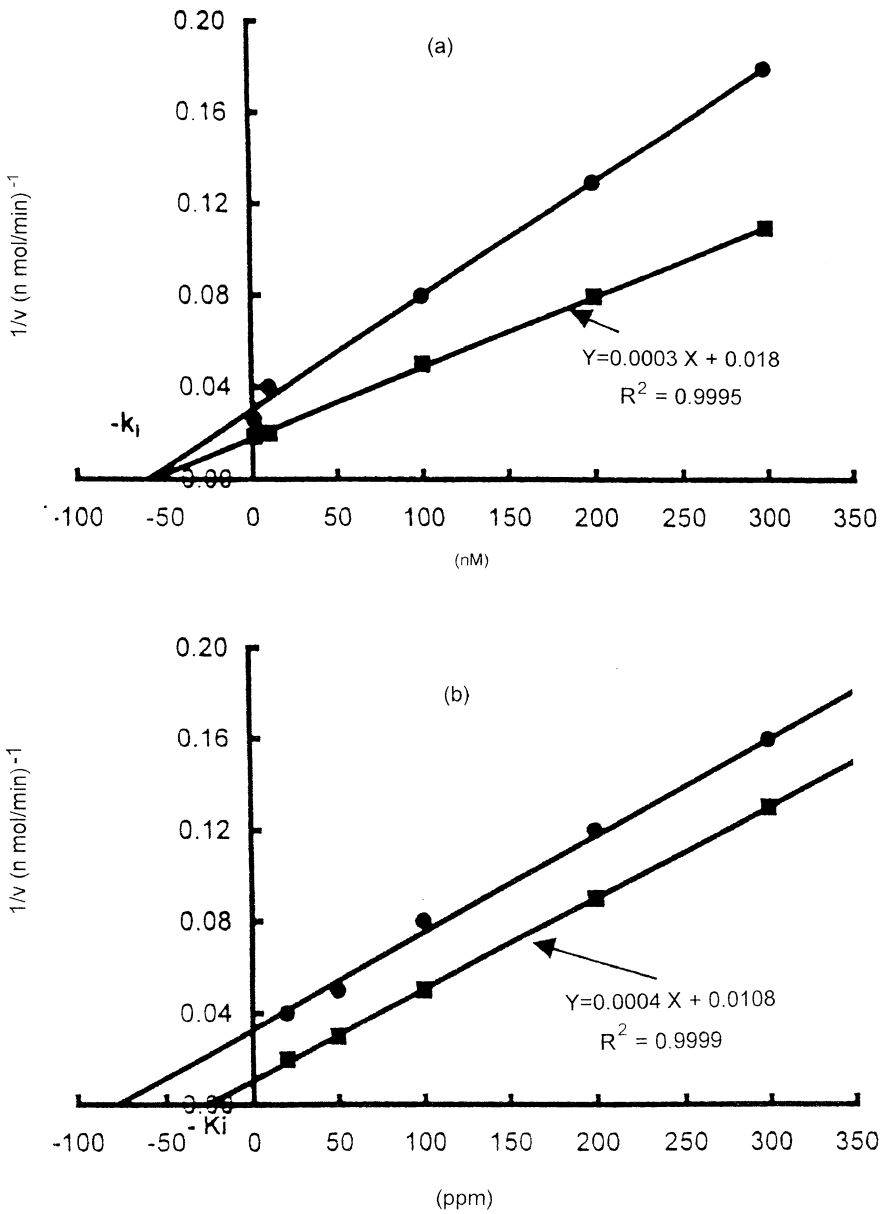

Fig. 7. Dixon plot: Effect of different concentrations of spinosad (a) and different concentrations of *B. bassiana* (b). Activity was determined in assay containing 1.5 mM of GSH and (•), 0.5 and (■) 1 mM of DNB.

As a whole, the results obtained in the present study suggest that the insect glutathione S-transferases are similar in their properties to those that have been studied in vertebrate organisms. This similarity across a wide evolutionary gap is striking. The glutathione S-transferase from midguts larvae of *S.littoralis* was inhibited by nanomolar concentrations of spinosad and *B.bassiana*. The inhibition pattern of spinosad was a noncompetitive, whereas the inhibition pattern of *B.bassiana* was an uncompetitive. A further biochemical investigation is needed to explain this interaction.

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الخصائص الأولية لإنزيم جلوتاثيون اس-ترنسفيريز المفصول من المعدة الوسطى ليرقات دودة ورق القطن وتثبيطه بواسطة سباينوساد وفطر *بيوفاريا باسينيا* باستخدام أرثو-داى نيتروبنزين كمادة تفاعل

فكري الشهاوي وضيف الله الراجحي

قسم وقاية النبات ، كلية الزراعة ، جامعة الملك سعود

الرياض ، المملكة العربية السعودية

(قدم للنشر في ٦ / ١١ / ١٤١٩ هـ وقبل في ١٤٢٠/٢/٢٣ هـ)

ملخص البحث : تمت دراسة الخصائص الأولية لإنزيم جلوتاثيون اس-ترانسفيريز المفصول من المعدة الوسطى ليرقات دودة ورق القطن وتثبيطه بواسطة سباينوساد والفطر *بيوفاريا باسينيا* باستخدام أرثو- داى نيتروبنزين كمادة تفاعل . وكانت أهم النتائج المتحصل عليها ما يلي :

١- وجد أن خصائص إنزيم جلوتاثيون اس-ترنسفيريز المفصول من المعدة الوسطى ليرقات دودة ورق القطن تتشابه مع خصائص الإنزيم المدروس سابقا في الفقاريات .

٢-تم تثبيط نشاط إنزيم جلوتاثيون اس-ترانسفيريز بواسطة سباينوساد وفطر *بيوفاريا باسينيا* وكانت قيم التركيز المثبط لـ ٥٠ ٪ من النشاط هى ١٥.٠٦ نانومولر (٤٩,٦٩ جزء في المليون) ، ٥٣,٢٦ جزء في المليون على التوالي .

٣- بينت الدراسة الحركية لإنزيم جلوتاثيون اس-ترانسفيريز أن قيم ثابت ميخائيل (K_m) والسرعة القصوى (V_{max}) تجاه مادة أرثو داى نيتروبنزين كانت ٠,٤٦ ملليمول و ٥٣,٤٨ نانومول / دقيقة . وتجاه مادة الجلوتاثيون ١,٤٣ ملليمول و ٨٧,٧٢ نانومول / دقيقة على التوالي .

٤- وجد أن قيم ثابت التثبيط (K_i) لكل من سباينوساد وفطر *بيوفاريا باسينيا* كانت ٢٠٠ و ٢٧ جزء في المليون على التوالي ، ولذلك فإن فطر *بيوفاريا باسينيا* له قدرة تثبيطية لنشاط إنزيم جلوتاثيون اس-ترنسفيريز أكبر من سباينوساد .

٥- وجد أن التأثير التثبيطي لسباينوساد ضد إنزيم جلوتاثيون اس-ترنسفيريز من النوع اللاتنافسي (noncompetitive) بينما التأثير التثبيطي لفطر *بيوفاريا باسينيا* من النوع غير التنافسي (uncompetitive) .