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Purification and Characterization of Glutathione-S-transferase from Diamondback Moth, *Plutella xylostella* L.[1]

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Abstract The glutathione-S-transferase (GSTase) had been suggested as a possible metabolic enzyme related to insecticide resistance in the diamondback moth (DBM), *Plutella xylostella* L.and other insects. Although the GSTase was studied in many insects, it had not been thoroughly investigated in the DBM. In this study, GSTase was purified either by the traditional protein purification method or the affinity chromatographic method. The affinity chromatographic method was proven to offer a much simpler purification procedure and resulted in higher yield and purer grade of GSTase. The purification factors of GSTase by the affinity chromatography are 32.1 and 24.9 for resistant and susceptible DBM strains. The total activity of GSTase is higher in the resistant DBM, which was agreed with the previous observation that the organophosphate-resistant DBM possessed 2—4 fold higher GSTase activity than the susceptible DBM. After affinity chromatographic purification, only a single protein band was obtained in the polyacrylamide gel electrophoresis study, and the GSTase activity staining indicated the active zone is close but not totally matched the location of protein band. The explanation is unknown and needs further investigation. The molecular weight of DBM larval GSTase was measured at 45,000—46,500 daltons compared to other standard calibration proteins in the gel filtration columns, and is similar to the molecular weight of GSTases in housefly.

Introduction

In the previous investigation of the diamondback moth (DBM), *Plutella xylostella* L., the glutathione-S-transferase (GSH-S-transferase or GSTase) was recognized as a possible metabolic enzyme for organophosphorus insecticides (Cheng et al., 1983 & 1984). This enzyme is involved in mercapturic acid formation by catalyzing the initial conjugation of xenobiotic compounds with GSH, and has been investigated in mammalian

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tissues as well as in insects (Clark et al., 1986; Cochrane et al ., 1987; Habig et al., 1974; Jakoby, 1978; Motoyama and Dauterman, 1972 & 1975; Motoyama et al., 1977 & 1980; Oppenoorth et al., 1979; Ottea and Plapp, 1984).

In the DBM, the activity of GSTase increases rapidly from the 3rd instar to the 4th. In order to establish the technique for further investigation of GSTase in relation to insect physiology and insecticide resistance, the technique for the purification and characterization of this enzyme is explored.

Materials and methods

Diamondback moth

Susceptible strain: the native I-lan (IL) strain was used, which was first collected in Tou-chen (TC) of I-lan county in 1984 and has been kept in the laboratory ever since as the susceptible standard.

Resistant strain: the resistant strain was collected from Hsi-hu (HH), where vegetables grew year round. The insects were collected in 1987 for testing, and has been proven with high multiple resistance to all traditional insecticides.

Chemicals

Substrate used for GSTase assay was 1, 2-dichloro-4-nitrobenzene (DCNB) obtained from Tokyo Kasei; glycine, sucrose, riboflavin, Tris (hydroxymethyl) aminomethane, reduced glutathione (GSH), sulfobromophthalein-S-glutathione-agarose (BSP-GSH-agarose), bovine serum albumin (BSA) , cytochrome C (mol. wt. =12,400 daltons), carbonic anhydrase (mol. wt. =29,000 daltons), alcohol dehydrogenase (mol. wt. =150,000 daltons), and beta-amylase(mol. wt. =200,000 daltons)from Sigma Chemical Co.; ethylenediaminetetraacetate disodium dihydrate (EDTA) from Wako Pure Chemical Industries, Ltd.; DEAE-cellulose (DE52) in pre-swollen grade from Whatman Inc.; Hydroxylapatite (Bio-Gel HTP) from Bio-Rad Laboratories; Sephadex G-100 and G-200 from Pharmacia Fine Chemicals; ammonium sulfate, ammonium peroxodisulfate, bromophenol blue, Coomassie Brilliant Blue R, acrylamide, N, N′ methylene-diacrylamide (Bis), and N, N, N, N-tetramethylene-diamine (Temed) from E. Merck.

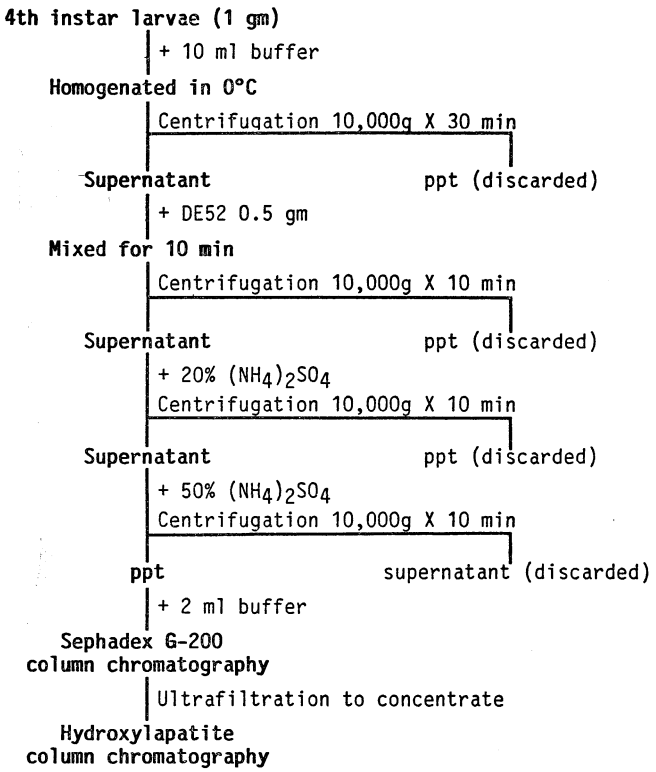
Enzyme assay

For the GSTase activity determination, DCNB was used as the standard substrate, and the spectrophotometric method of Booth et al. (1961) was employed. The change in absorbance at 344nm was converted to μmoles using the extinction coefficient ($e = 10nM^{-1}\ cm^{-1}$) for S-(2-chloro-4-nitrophenyl)-glutathione (Askelof et al., 1975).

Purification of GSTase

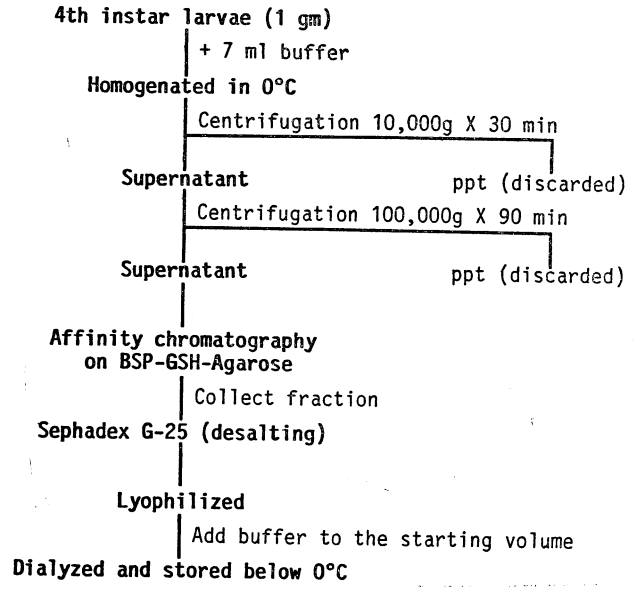
The purification of GSTase from DBM larvae followed two methods: (1) the traditional ammonium sulfate fractionation combined with column chromatography (Scheme 1) described by Motoyama and Dauterman (1977 & 1978) and (2) the newly developed affinity chromatography method (Scheme 2) described by Clark et al. (1977), Simons and Vander Jagt (1977), and Cochrane et al. (1977). Scheme 1 and 2 illustrated the detailed purification steps, and some modifications from previous work in housefly or *Drosophila* spp. were described later in this report. The isolation buffer used for GST-

Scheme 1. Purification of GSTase by traditional method.



ase is 0.01M potassium phosphate(pH 7.4)containing 2mM GSH and 0.1mM EDTA. The specification for Sephadex G-200 column is 2cm (dia.) ×53cm (height), and 1.5 to 1.9ml per tube were fractionated.

Scheme 2. Purification of GSTase by affinity chromatography



The specification is 1.0cm (dia.) ×14.5cm in length for HTP chromatographic column, while the linear gradient from 150ml isolation buffer and 150ml 0.4M potassium phosphate buffer containing the same concentration of GSH and EDTA as the isolation buffer were used to exchange GSTase. The fractions were collected as 5ml per tube.

Molecular weight determination

The GSTase purified from Scheme 1 was loaded in a Sephadex G-100 gel filtration column with the known protein standards and eluted in two buffer systems as they were 0.05M Tris buffer (pH 8.0) and 0.01M phosphate buffer (pH 6.7). The elute was monitored in 280nm for protein peaks. Generally, the methods of Andrews (1964) and Motoyama and Dauterman (1979) were followed.

Electrophoresis

The polyacrylamide gel electrophoresis (PAGE) of Scheme 2 purified GSTase sample in contrast to the crude preparation was performed according to Gordon (1975), Hames and Rickwood(1981). The pH of running gel buffer was 8.9. Protein was stained with Coomassie Brilliant Blue R; GSTase activity was stained with the same enzyme assay solution using DCNB as substrate in 37°C for 30—50min and then scanned at 344nm for absorbance.

Protein determination

Protein was determined by the Lowry method (1951) with Folin-Ciocalteu's phenol reagent, and using BSA as protein standard.

Results and discussions

GSTase purification

Some modifications in the traditional protein purification method (Scheme 1) used in the housefly GSTase study (Motoyama and Dauterman, 1977 & 1978) had been adopted in the very beginning of DBM's GSTase purification. In the housefly preparation,

Table 1. The effect of buffer volume added per gm of *P. xylostella* larvae and 0.5gm DE52 in relation to the initial recovery of GSTase activity in purification.

Buffer volume per gm *P. xylostella* larvae and 0.5gm DE52	Recovered GSTase from 10,000g supernatant
2ml	30-48%
3ml	65%
5ml	86%
10ml	98%

the buffer volume is 200ml for 95gm insects, while the same dilution for the DBM resulted in great GSTase activity loss in the DEAE-cellulose treatment. By raising the buffer: insect ratio to 10 : 1 (V/W), the recovery of GSTase activiey reached 98% (Table 1). Another major change is the omission of glycerol and BSA from the purification buffer since no detectable GSTase activity loss could be found by the above mentioned

omission. The purified GSTase sample without glycerol can be easily lyophilized for storage and adjusted to desirable concentration by redissolving in suitable amount of buffer whenever needed.

Table 2. Purification of GSTase from S-strain *P. xylostella* followed the procedure of scheme 1.

Procedure	Total units* per gm DBM	Yield (%)	Specific activity**	Purification (fold)
1. 10,000g sup.	523.8	100	4.92	1.00
2. DEAE-cellulose	510.7	97	14.46	2.94
3. AS 20—50% ppt	458.5	88	25.97	5.28
4. Sephadex G—200	406.8	78	37.50	7.62
5. Hydroxylapatite	175.7	34	54.50	11.08

* Unit of activity is defined as 1 μmole of DCNB conjugated with GSH per min at 25°C.
**Specific activity is defined as 1 unit of GSTase activity per mg protein.

The GSTase purification followed Scheme 1 is presented in Table 2. As we had reported before that the 4th instar DBM larvae posses high GSTase activity compared to the housefly (Motoyama and Dauterman, 1978). With the exception of the DEAE-cellulose step, the purification of all other steps were not as good as that in the house fly study. Up to the step of hydroxylapatite column chromatography, purification is only 11.08 fold compared to 69 fold in the housefly. The recovery is 34% compared to 55% in housefly. Since no major protein impurity interfered as that in the housfly, the second hydroxylapatite chromatography was not performed.

Table 3. Purification of GSTase from R- and S-strain *P. xylostella* larvae followed scheme 2.

Procedure	(Per gm) R-strain DBM*				(Per gm) S-strain DBM*			
	Total units**	Rec. (%)	Specific activity**	P. F.	Total units**	Rec. (%)	Specific activity.***	P. F.
1. 10,000g sup.	883.1±88.3	100	12.6±0.7	1.0	451.1±42.8	100	8.5±2.5	1.0
2. 100,000g sup.	939.1±74.7	106	17.8±2.2	1.3	412.3±37.4	91	12.1±2.3	1.4
3. Aff. chrom	404.7±29.7	46	435.9±37.5	32.1	253.5±12.3	56	211.3±28.4	24.9

*The 4th instar larvae were used. With 4 and 3 replicateds for R-and S-strain, respectively.
**Unit of activity is defined as 1 μmole of DCNB conjugated with GSH per min. at 25°C.
***Specific activity is defined as 1 unit of GSTase activity per mg protein.
P. F. =Purification factor.

The purification of GSTase by affinity chromatography consists of only three major steps (Scheme 2 and Table 3), and the purification factors were 32.1 and 24.9 in R- and S-strain of DBM, respectively. Nevertheless, the affinity chromatography can be considered as one step purification in the whole scheme, since high speed centrifugation only offered minimum improvement as the purification factors were between 1.3—

Fig. 1. Electrophoresis of affinity chromatography purified GSTase from *P. xylostella* L.
a. sample after 10,000 and 100,000g centrifugation.
b. sample after BSP-GSH-agarose affinity chromatography.

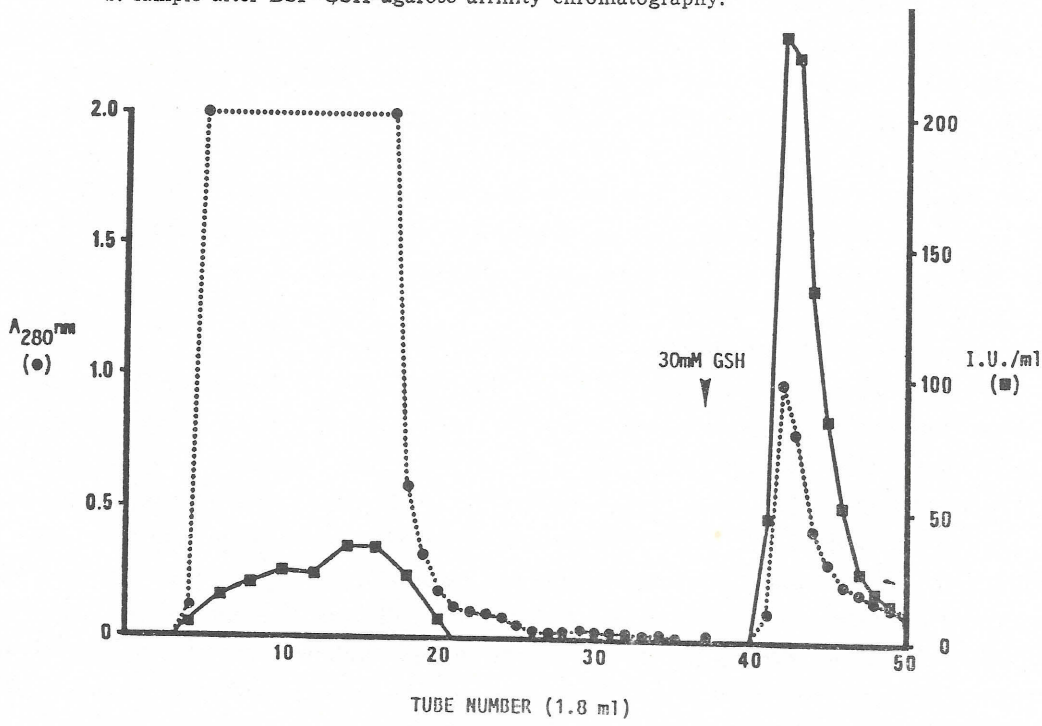


Fig. 2. (a) Profile for the purification of GSTase from S-DBM using BSP-GSH-affinity chromatography.

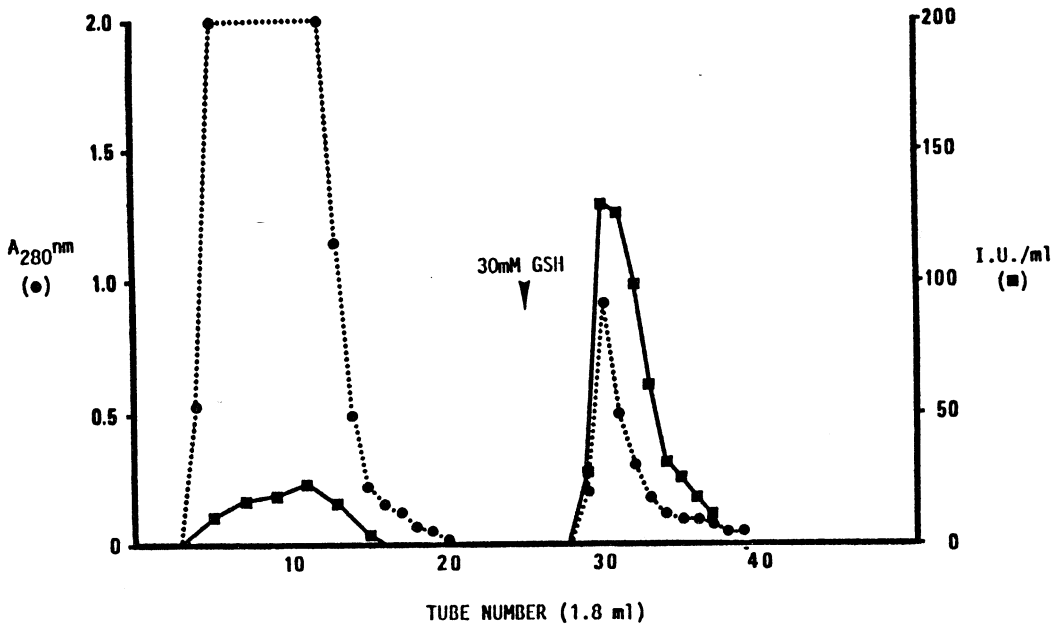


Fig. 2. (b) Profile for the purification of GSTase from R-DBM using BSP-GSH-affinity chromatography.

1.4. The 46—56% yield in GSTase is also better when compared to 34% of the scheme 1 traditional method. Furthermore, the polyacrylamide gel electrophoresis study showed that the affinity chromatography is so specific that only one major protein band could be recognized after staining (Figure 1), which demonstrated the affinity chromatography method is a better choice for GSTase purification in the DBM. Instead of 5mM used in *Drosophila* spp. (Cochrane et al., 1987) and human liver (Simons and Vander Jagt, 1977), 30mM GSH is needed to elute DBM GSTase from the BSP-GSH-agarose column for DBM. The BSP- GSH-agarose affinity chromatography profile for the purification of GSTases from S- and R-strain of DBM were presented in Figure 2a and 2b, and there were 25—27% GSTase activity loss in loading and subsequent buffer washing. Main GSTase activity recovery was obtained right after the introduction of 30mM GSH into the elution buffer. Both profiles were rather similar to that of human liver GSTase reported by Simons and Vander Jagt (1977).

Characterization of the molecular weight of DBM GSTase

The elution character of DBM's GSTase from Sephadex G-200 column in contrast to the protein elution profile is rather similar to the results of housefly (Motoyama and Dauterman, 1978) except the GSTase of DBM can be successfully discriminated from the second major protein peak (Figure 3). In the subsequent hydroxylapatite column chromatography, the sample was eluted without the major protein peak presented (Figure 4). Nevertheless, the needed potassium phosphate concentration to exchange the enzyme was extremely similar to that of housefly's GSTase. The molecular weight of DBM's GSTase measured was between 45,000—46,500 daltons (Figure 5a and 5b) in contrast to 37,000—54,000 daltons (Motoyama and Dauterman, 1978) and 43,000, 44,600 and 53,600 daltons (Motoyama and Dauterman, 1979).

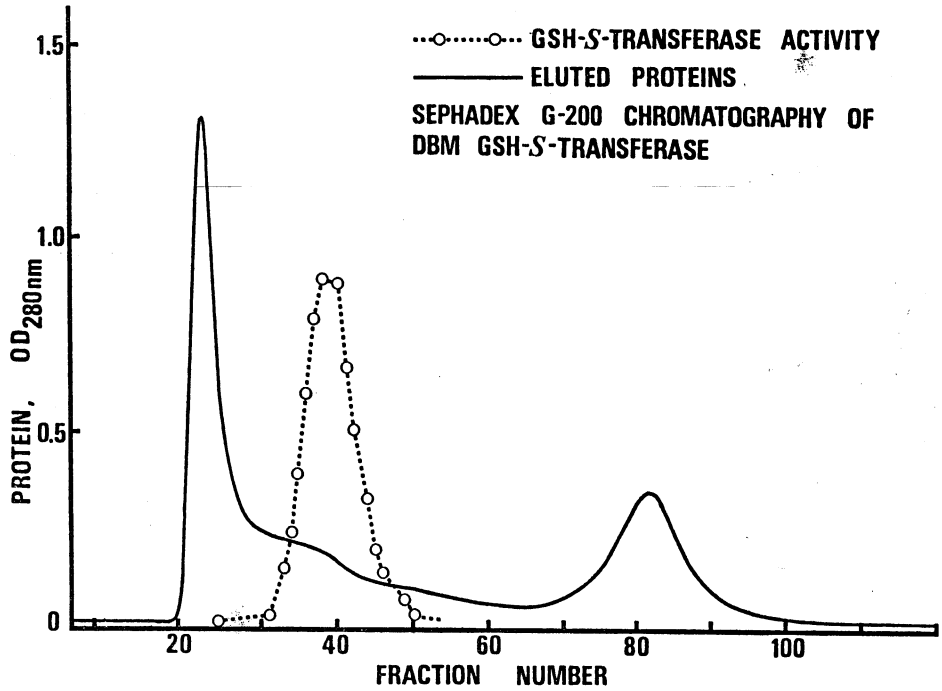


Fig. 3. Sephadex G-200 chromatography of GSTase from S-DBM in purification Scheme 1.

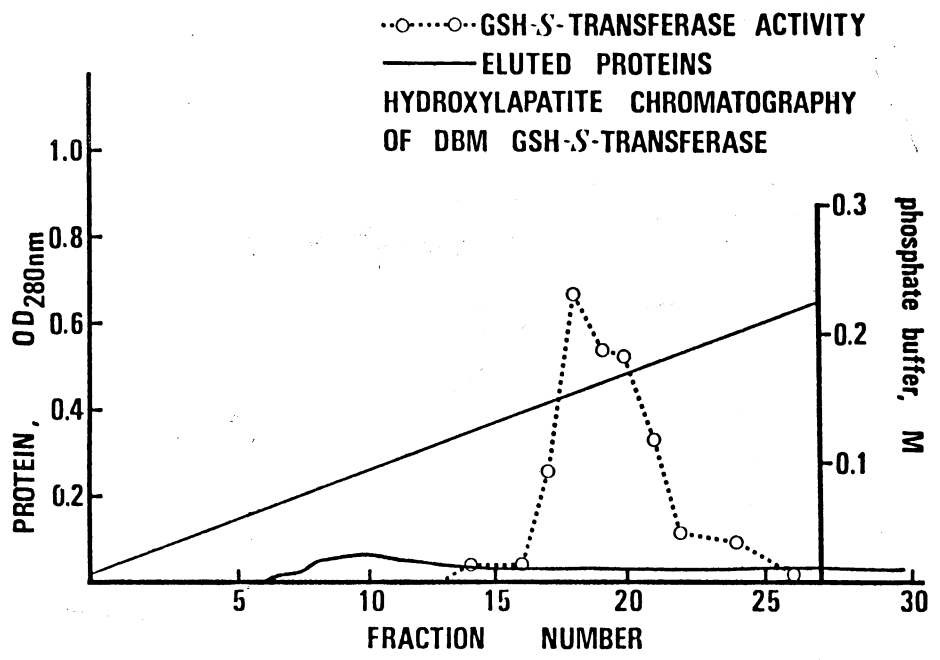


Fig. 4. Hydroxylapatite chromatography of GSTase from S-DBM in purification Scheme 2.

Electrophoresis and staining of GSTase

DBM's GSTase sample obtained from Scheme 1 procedure still contained many protein impurities in polyacrylamide gel electrophoresis, hence only the sample purified from the affinity chromatography was investigated further (Figure 1). The crude GSTase preparation served as the comparative check. The staining method for GSTase activity described by Clark(1982)had been tested but no satisfactory result could be obtained, hence a different GSTase activity staining method was developed. After electrophoresis, the gel was rinsed in 0.1M Tris buffer (pH 9.0) and then soaked in 50ml of 0.1M Tris buffer (pH 9.0) containing 1mM DCNB and 5mM GSH in 37°C for 50 minutes. After incubation, the gel was transferred into a densitometer and scanned at 344nm for absorbance reading. A strong single absorbance peak was located at Rf=0.06 to 0.21 (Figure 6) while the protein stain of the same gel revealed the single protein band located at Rf=0.06—0.11 (Figure 1). The reason for the GSTase activity peak and the protein location were close but not exactly matched is not clear and needs to be investigated further. However, the PAGE result definitely demonstrates that affinity chromatography has superior GSTase purification capability when compared to the traditional method.

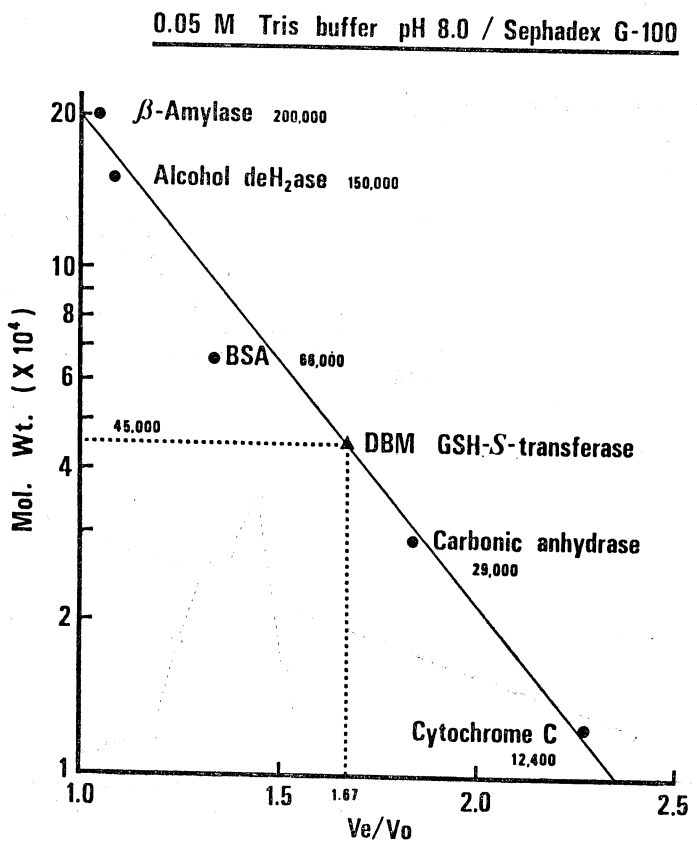


Fig. 3. (a) Molecular weight determination of GSTase in Sephadex G-100 gel filtration column with 0.05M Tris buffer (pH=8.0). Enzyme source: S-DBM inpurification Scheme 1.

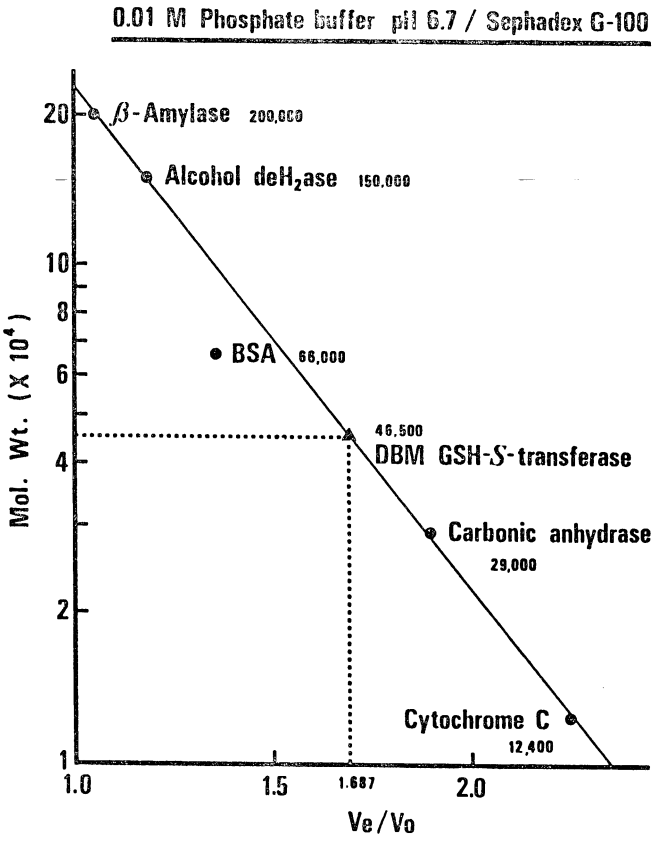


Fig. 5. (b) Molecular weight determination of GS Tase in Sephadex G-100 gel filtration column with 0.01 M phosphate buffer(pH6.7). Enzyme source: S-DBM inpurification Scheme 1.

GSTase in relation to insecticide resistance in the DBM

In previous report, the organophosphate-resistant DBM possessed higher GSTase activity compared to both the susceptible and pyrethroid-resistant DBMs, and the difference in enzyme activity was approximately 2 to 4 fold (Cheng et al., 1983). In this investigation, the field collected multiple resistant DBM also possessed 2 fold GSTase activity in contrast to the susceptible DBM. The experiment had been repeated 3 to 4 times, and similar difference appeared every time. The higher GSTase activity in the resistant DBM was attributed from both enzyme protein and the specific activity (Table 3). Although direct evidence to relate GSTase to the organophosphate resistance in the DBM is still lacking, the consistant appearance of high GSTase activity in the resistant DBM strongly suggested the possibility of GSTase being an organophosphate metabolic enzyme, and may contribute to the resistance.

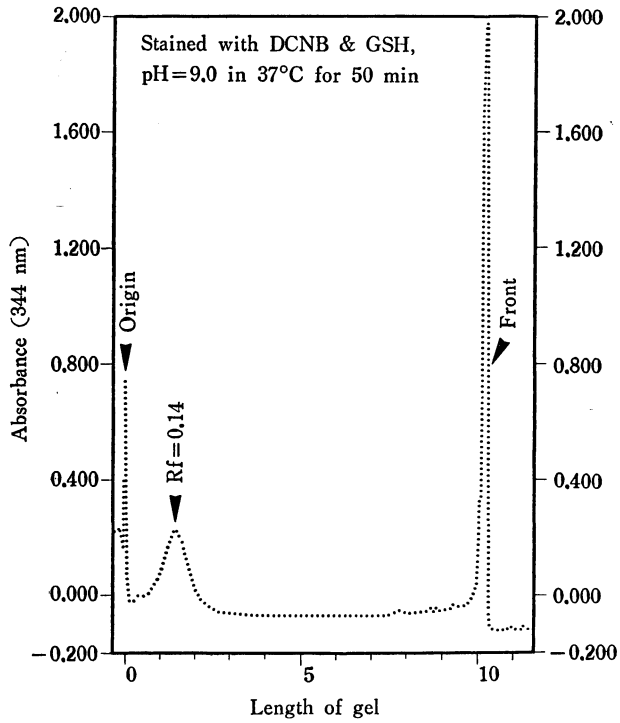


Fig. 6. Densitometer scan of GSTase activity in electrophoresis gel. (substrate: DCNB, incubation temperature:37°C, scanning absorbance:344nm.)

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小菜蛾Glutathione-S-轉移酶之純化與定性[1]

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摘 要

Glutathione-S-轉移酶在小菜蛾以及其他昆蟲體內，被認爲可能與抗藥性有關，尤其是有機磷殺蟲劑中之甲基或乙基的解裂，而使殺蟲劑在到達毒理作用點以前因分解而失效。小菜蛾之抗藥性極困擾蔬菜之蟲害防治，而有關有機磷抗性之機制，迄今仍不完全瞭解，故就此蟲之 Glutathione-S-轉移酶進行純化與定性。本研究以兩種方式進行該酶之純化，一爲傳統之「鹽析」及「分子篩分離」以及「離子交換」方法，一爲利用新發展之「親和性分析柱」方式，結果發現後者之純化步驟簡單，回收率及酶之純度均較傳統之純化方法爲佳，純化倍數爲 25—32倍之間，同時測得小菜蛾之 Glutathione-S-轉移酶分子量介於 45,000～46,500 daltons 之間，而且，此酶之活性在抗性品系小菜蛾高過感性品系 2 倍以上。

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