Induction of Glutathione S-Transferase Isozymes in Rice Shoots Treated with a Combination of Pretilachlor and Fenclorim*

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Abstract: DEAE Sephacel anion exchange chromatography was used to separate glutathione S-transferase (GST) isozymes in non-treated etiolated rice shoots (4 day-old) and those treated with a combination of pretilachlor [2-chloro-2',6'-diethyl-N-(2-propoxyethyl)-acetanilide] and fenclorim (4, 6-dichloro-2-phenylpyrimidine).

Non-treated rice shoots contained isozymes which were active with the following substrates; CDNB: 1-chloro-2,4-dinitrobenzene (2 isozymes), fenclorim (2 isozymes) and pretilachlor (4 isozymes). Pretreatment of the shoots with a combination of pretilachlor and fenclorim increased the activity of the constitutively-expressed isozymes that exhibited activity with CDNB, fenclorim and pretilachlor. The treatment also induced two new GST(fen) isozymes and one new GST(pret) isozyme. The results are suggestive of the diversity of GST isozymes in etiolated rice shoots, the selective enhancement of GST isozymes by herbicides and safeners, and safeners conjugated with glutathione, resulting in selective safening action in rice.

Key words: rice, glutathione S-transferase (GST) isozymes, pretilachlor, fenclorim

Abbreviations: GST(CDNB), glutathione S-transferase utilizing CDNB as a substrate; GST(fen), glutathione S-transferase utilizing fenclorim as a substrate; GST(pret), glutathione S-transferase utilizing pretilachlor as a substrate.

Introduction

Pretilachlor is a chloroacetamide herbicide commonly used to control certain broad leaved weeds and annual grasses in transplanted rice fields1,2,11). Its selectivity between rice and grass weeds was reported to relate to metabolism of the herbicide mediated by glutathione S-transferase (EC 2.5.1.18; GST) activity4,5,12,13,17,24). However, this selective mechanism of pretilachlor has not been completely identified.

Fenclorim is a safener used to protect rice from pretilachlor injury1,22). Although the safening mechanism of this herbicide safener has not yet been elucidated, there is evidence that fenclorim could enhance herbicide detoxification by conjugation with reduced glutathione (GSH) in rice plant.

GSTs are multifunctional dimeric enzymes which can conjugate GSH with electrophilic compounds including chloroacetamide, thiocarbamate and triazine herbicides11,16,18,25). There are various reports on the existence of many GST isozymes in ani-

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and it has been demonstrated that there are at least 4, 5, 2 and 7 isozymes of GST in corn, chickpea, pea and sorghum, respectively. GSTs are also induced by a diversity of xenobiotic chemicals including herbicides and herbicide safeners. Sorghum and corn treated with a safener or an herbicide show elevated levels of total GST activity and induce new GST isozymes, which subsequently result in the enhanced metabolism of metolachlor to its GSH conjugate. It is well established that the GST isozymes in corn or sorghum differ in substrate specificity, but it has not been determined whether such isozymes in rice and fenclorim confer protection by inducing GST isozymes which exhibit high reactivity toward the chloroacetamide herbicide pretilachlor.

The objectives of this study were to determine: (a) the existence of GST isozymes and their substrate specificities, and (b) the effect of pretilachlor and fenclorim on GST induction in rice shoots.

Materials and Methods

Chemicals

Pretilachlor and fenclorim (Fig. 1) with a purity of 96% each were provided by Ciba-Geigy Corporation (Japan). All other chemicals were purchased.

Plant materials

Rice (Oryza sativa L. cv. Nipponbare)

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Fig. 1. Chemical structures of CDNB, fenclorim and pretilachlor and position of their GSH conjugation in circlet.
Enzyme activities

For GST\(_{(CDNB)}\) activity, the reaction mixture contained 500 \(\mu\)L chromatography fraction (enzyme extract), 500 \(\mu\)L of 0.1 M K-phosphate (pH 7.4), 450 \(\mu\)L of 3.3 mM GSH and 50 \(\mu\)L of 30 mM CDNB in ethanol. The reaction was started by addition of substrates and then the OD change was monitored at 340 nm and 25°C by a spectrophotometer\(^{10}\). GST\(_{(CDNB)}\) activity values were corrected by subtraction of non-enzymic conjugation.

For GST\(_{(fen)}\) activity, 500 \(\mu\)L chromatography fraction was mixed with 100 \(\mu\)L of 60 mM GSH and 8 \(\mu\)L of 25 mM fenclorim in methanol. The reaction mixture was incubated for 1 hour at 30°C and then 5 mL of ice-cold dichloromethane was added to terminate the reaction. The amount of GS-fenclorim conjugate formed in aqueous phase was determined by HPLC\(^6\). The running system for HPLC procedure was as follows: mobile phase, water/methanol (55:45) containing 5 mM KH\(_2\)PO\(_4\); flow rate, 1.2 mL/min; detection wavelength, 262 nm; column, Shim pack-phenyl (\(\Phi\)4.6 mm x 150 mm).

GST\(_{(pret)}\) activity was measured by monitoring the GS-conjugate formed with HPLC\(^4\). The reaction mixture contained 500 \(\mu\)L chromatography fraction and 100 \(\mu\)L of 60 mM reduced glutathione. The reaction was initiated by adding 8 \(\mu\)L of 25 mM pretilachlor. After incubation at 30°C for 60 min, the reaction was terminated by adding 5 mL dichloromethane. The aqueous phase was then analyzed with HPLC to determine the amount of GS-conjugate. Mobile phase: water/methanol (48:52) containing 5 mM KH\(_2\)PO\(_4\); flow rate, 1.2 mL/min; detection wavelength, 210 nm; column, Shim pack-phenyl (\(\Phi\)4.6 mm x 150 mm). The rate of the enzymatic conjugation with glutathione was calculated from the data obtained from the crude enzyme extract by subtracting the nonenzymatic rate in the absence of the extract.

Results

CDNB, fenclorim and pretilachlor were used as substrates to detect the presence of GST activity following DEAE-Sephadex anion exchange chromatography of crude extracts from shoots of non-treated rice
seedlings (Fig. 2).

The non-treated shoots of rice showed two peaks of GST\textsubscript{(CDNB)} (peak II, III), two peaks of GST\textsubscript{(fen)} (peak IV, VI) and at least four peaks of GST\textsubscript{(pret)} (peak I, V, VII, VIII).

The DEAE Sephacel anion exchange elution profiles of GST activity in etiolated rice shoots treated with pretilachlor and fenclorim are shown in Fig. 3. The activity of GST\textsubscript{(CDNB)} increased ca. 2.5- and 1.4-fold in peak II and III, respectively. GST\textsubscript{(fen)} activity of peak IV and VI also increased approximately 1.4- and 1.7-fold, and GST\textsubscript{(pret)} activity peak of I, V, VII and VIII increased approximately 4.0-, 3.9-, 4.5- and 21.5-fold over the same peak in the non-treated tissue, respectively. At least two new peaks of GST\textsubscript{(fen)} activity (peak IX, X) and one new peak of GST\textsubscript{(pret)} activity (peak XI) were also induced.

**Discussion**

DEAE Sephacel anion-exchange chromatography proved to be an effective method for separation GST isozymes in extracts of etiolated rice shoots. The peaks observed in the elution profiles are thought to represent distinct isozymes. On the basis of activity with herbicide pretilachlor, safener fenclorim and CDNB, a general substrate for GSTs, it is apparent that both non-treated and treated etiolated rice shoots contained several different GST isozymes. In this study, pretilachlor appeared to be a better model substrate of GST than fenclorim, but a poorer one than CDNB. This tendency was identified by the case of total crude GST activity in rice. Isozymes of GST in rice had different substrate specificities similar to those of corn and sorghum\textsuperscript{2,3}. On the basis of reactivity with three substrates, at least eight and eleven peaks of GST activity were detected in non-treated and treated shoots, respectively, but the exact number and characteristics of isozymes is yet to be determined.

In non-treated rice shoots, there were two constitutive GST\textsubscript{(CDNB)} isozymes (II, III), two for GST\textsubscript{(fen)} (IV, VI) and four for GST\textsubscript{(pret)} (I, V, VII, VIII). This finding is the first report of GST isozymes having activities toward CDNB, fenclorim and pretilachlor in rice. We previously reported that GST\textsubscript{(fen)} and GST\textsubscript{(pret)} had different optimum pH 8.4 and

![Fig. 3. DEAE Sephacel anion exchange chromatograms of the activities of GST using various substrates from shoots of etiolated (4 day old) rice treated with pretilachlor and fenclorim. *GST\textsubscript{(pret)}, GST\textsubscript{(fen)}: pmol/ml/min; GST\textsubscript{(CDNB)}: nmol/ml/min.](image_url)
Dean et al. reported that corn contained one, two and three constitutive GST isozymes which had activity toward CDNB, metolachlor and atrazine, respectively. They also reported that sorghum contained only one constitutive GST isozyme for CDNB and herbicide metolachlor. In this study, 3 GST\textsubscript{CDNB}, 3 GST\textsubscript{fen} and 6 GST\textsubscript{pret} isozymes were identified from 1-leaf-stage seedlings (data not shown). The number and activities of GST isozymes are believed to be dependent on growth age and the condition of the rice plant. This result was similar to the previous studies in which GST isozyme activities appeared to be dependent on culture term in corn. The number and activities of GST isozyme were confirmed to relate to plant species, culture term, substrate and growth condition.

In pretilachlor and fenclozim treated rice shoots, on the other hand, constitutive GST\textsubscript{CDNB}, GST\textsubscript{fen} and GST\textsubscript{pret} isozyme activities increased. This result clearly confirmed that treatment with a combination of pretilachlor and fenclozim caused a selective enhancement of GSTs in etiolated rice shoots. In this treatment, two constitutive GST\textsubscript{CDNB} (II, III), two constitutive GST\textsubscript{fen} (IV, VI) and four constitutive GST\textsubscript{pret} (I, V, VII, VIII) isozyme activities increased. Two new GST\textsubscript{fen} (IX, X), one new GST\textsubscript{pret} (XI) were induced but no GST\textsubscript{CDNB}. This was similar to the case of sorghum in which GST\textsubscript{CDNB} isozyme activity increased, but no new isozyme was observed in the treatment either with herbicide metolachlor or a safener such as dichlormid or flurazole. However, in corn, a constitutive GST\textsubscript{CDNB} isozyme activity increased and a new isozyme was induced following safener benoxacor (CGA-154281) treatment.

An earlier study indicated that the safeners dichlormid and flurazole were converted to GSH conjugates in corn shoots, although it was not known whether or not this reaction was catalyzed by GST. We demonstrated that the reaction of fenclozim with GSH was catalyzed by GST\textsubscript{ten} in rice plant and also reported that the activity of crude GST\textsubscript{ten} enzyme in this plant was induced following treatment with pretilachlor and fenclozim. The present study demonstrates that two constitutive GST\textsubscript{ten} isozyme activities increased and two new GST\textsubscript{ten} isozymes were induced in rice shoots treated with the combination of pretilachlor and fenclozim. GST isozyme activities using CDNB as substrate were discovered not only in gramineae such as corn, wheat, rice and sorghum, but also in woody plants. Moreover, three GST\textsubscript{CDNB} isozymes were observed in 1-leaf-stage rice seedlings (data not shown) and needles of mature (80- to 140-yr-old) Norway spruce (Picea abies) trees, respectively. In this report, there seemed to be a 3rd peak of GST\textsubscript{CDNB} (isozye) activity in the 100 to 120 ml elution profile, but the activity was sparse, and the peak could not be separated clearly by DEAE Sephacel chromatography in non-treated or treated rice shoots (Fig. 2, 3). Further investigation of this is needed in future.

In conclusion, from the above results four constitutive GST\textsubscript{pret} isozymes increased and one new isozyme was induced in treated rice shoots. This may support out earlier findings indicating that the GST\textsubscript{pret} activity and Vmax of GST\textsubscript{pret} increased and Km of GST\textsubscript{pret} decreased after rice seedlings were treated with pretilachlor and/or fenclozim. It suggests that induced GST\textsubscript{pret} isozyme has higher affinity to pretilachlor than its constitutive isozyme in rice shoots. Moreover, it was also evident that resistance of rice to pretilachlor and the safening effect of fenclozim may be related to the induction of
GST(pret).

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**Deng et al. : GST Isozyme Induction in Rice**

ブレチラクロールとフェンクロリムの混合処理 
によるイネにおけるグルタチオン S-トランスフェ 
ラーゼアイソザイムの誘導

**摘 要**

無処理及びブレチラクロールとフェンクロリムの 
混合処理を24時間処理した黄化イネの茎葉部における 
CDNB, フェンクロリム, ブレチラクロールをそれ 
ぞれ基質とするGST(CDNB), GST(ten)及びGST(pret)ア 
イソザイムについて, DEAE-Sephacel カラムの分 
離パターンにより検討した。

無処理区のイネ茎葉部から抽出したGSTには, 2 
つのGST(CDNB), 2つGST(ten)及び4つGST(pret) 
アイソザイムがあると推定される (Fig. 2)。これら 
のアイソザイムの活性及び溶媒パターンが異なったこ 
とから, CDNB, フェンクロリム, ブレチラクロー 
ルに対する基質特異性の異なるGST の存在が確認さ 
れた。

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ブレチラクロールとフェンクロリムを混合処理し 
たイネにおいて(Fig. 3), CDNB を基質とした場合, 
本来から存在したピーク II, IIIの2つのアイソザイ 
ムの活性が2倍近く増加し, GST(CDNB)の誘導が見ら 
れた。フェンクロリムを基質とした場合にも, もと 
も存在したピークIV, VIの活性が増加し, また, 
ピークIX, Xが新たに誘導され, 二つのGST(teen)ア 
イソザイムが誘導されたと考えられる。ブレチラクロ 
ールを基質とした場合は, もともとピーク I, V, 
VII, VIIIの活性が増加し, またピーク XIが新たに誘導 
され, 1つ新しいGST(pret)アイソザイムが誘導され 
たと見られる。

これらの結果から, イネにおけるGST には, ブレ 
チラクロール, フェンクロリム, CDNB に対し, 基 
質特異性の高い数種のアイソザイムが存在すること 
が確認された。また, イネのブレチラクロールに対 
する抵抗性及びフェンクロリムの薬害軽減効果は, 
GST 活性及びその誘導と深く関連していることが示 
唆される。

**キーワード:** イネ, グルタチオン S-トランスフェ 
ラーゼ (GST) アイソザイム, ブレチラクロール, 
フェンクロリム