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Induction of Glutathione Metabolism in the Fat Body of *Sarcophaga peregrina* by Integumental Injury[1]

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When the body wall of *Sarcophaga* larvae was pricked to activate various defense protein genes, the glutathione level in the fat body was found to decrease significantly. Previously, we showed that these defense protein genes were activated when the fat body was incubated in buffered insect saline [Sugiyama and Natori (1991) *Eur. J. Biochem.* 200, 495–500]. Under these conditions, the glutathione level was also found to decrease significantly. Probably, the decrease of glutathione level is crucial for activation of insect NF-κB-like factor, which has been suggested to participate in the expression of various defense protein genes.

Key words: defense protein genes, glutathione, insect, NF-κB, *Sarcophaga* lectin.

Insects have developed a unique defense system against bacteria and other parasites consisting of various inducible defense molecules such as antibacterial proteins and lectins (*1-9*). The genes for these proteins are activated when live or dead bacteria are injected into insects or when their integument is injured (*10-12*). These defense proteins are mainly produced in the fat body, which is equivalent to the mammalian liver, but some are produced by hemocytes (*13*). Cultured cell lines from dipteran insects can now be used to study the mechanism of activation of defense protein genes (*14, 15*), and recent results have suggested that transcription factors such as an equivalent of mammalian NF-κB are involved in the expression of insect defense protein genes (*16*).

In *Sarcophaga peregrina* (flesh fly) larvae, the genes for various defense proteins are activated simultaneously in the fat body when the body wall is pricked with a hypodermic needle. Previously, we reported that the genes for *Sarcophaga* defense proteins such as *Sarcophaga* lectin, sarcotoxin IA, and sarcotoxin IIA were activated when larval fat body was simply incubated in buffered insect saline *in vitro* (*17*). We analyzed the mechanism of activation of these genes, and found that treatment of the fat body with 2-mercaptoethanol completely abolished the activations of these genes, suggesting that oxidation of the sulfhydryl group of a fat body protein is required for their activation. Moreover, we found that the oxidation reaction of the fat body protein, if any, occurs at an initial stage of incubation of the fat body and that once this reaction is completed, subsequent transcription takes place even in the presence of 2-mercaptoethanol. We also found that the amount of sulfhydryl groups in fat body protein decrease drastically when the fat body was incubated in buffered insect saline *in vitro*.

In this paper, we demonstrated that the main substance with active sulfhydryl groups in the fat body of *Sarcophaga* larvae is glutathione, and that its content in the fat body decreased transiently when the larval body wall was injured. These findings suggest that the decrease of glutathione level in the fat body is crucial for the activation of the defense protein genes.

MATERIALS AND METHODS

Animals and Fat Body—Flesh flies, *S. peregrina*, were kept at 27°C with dry milk, sugar cubes, and fresh water as reported before. Larvae were reared on pork liver, and when they crawled upward at the third instar, they were collected, washed well and kept in a plastic container with a small amount of water. Before injury of the body wall, larvae were anesthetized by placing them on a glass plate in ice for a few minutes. Then the posterior half of their body wall was pricked with a hypodermic needle. Larvae could be used for experiments for up to 3 days after leaving their food. Fat body was excised from third instar larvae under a binocular microscope and rinsed well in ice-cold insect saline (130 mM NaCl, 5 mM KCl, and 1 mM $CaCl_2$).

Assay of Active Sulfhydryl Groups in Fat Body Protein—This assay was done essentially by the method of Grassetti and Murray (*18*). Briefly, 2,2′-dithiodipyridine was dissolved in phosphate-buffered insect saline (10 mM NaH_2PO_4/Na_2HPO_4, 2 mM $NaHCO_3$, 1 mM $MgCl_2$, 5 mM KCl, 1 mM $CaCl_2$, and 120 mM NaCl) at a final concentration of 0.1 mM and the solution was adjusted to pH 6.2. A fat body was immersed in 1 ml of this solution and kept for 2 min at 4°C with gentle stirring. The fat body was then removed, and the absorbance at 343 nm of the medium was

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Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; GSH, glutathione; GSSG, glutathione disulfide; mBBr, monobromobimane; SH, sulfhydryl; TCA, trichloroacetic acid.

measured to determine the amount of 2-thiopyridone formed in the medium as a measure of the content of sulfhydryl groups in the fat body protein. The protein content of the fat body was measured by the method of Lowry *et al.* (*19*). The reaction time of the fat body and 2,2′-dithiodipyridine was fixed at 2 min to measure the amount of sulfhydryl groups that were preferentially susceptible to oxidation.

Labeling with [^3H]-N-Ethylmaleimide and Subcellular Fractionation of the Fat Body—Fat body was treated with 1 ml of phosphate-buffered insect saline containing 0.1 mM *N*-ethylmaleimide (NEM) and 20 μCi of [^{3}H]NEM for 2 min at 4°C to label active sulfhydryl groups. Then the fat body was washed well, transferred into 10 mM NEM solution and kept for 30 min at 4°C. It was homogenized in 1 ml of 10 mM Tris/HCl buffer pH 7.4, containing 0.25 M sucrose, 5 mM 2-mercaptoethanol, 2 mM EDTA, 50 μg/ml of leupeptin, 50 μg/ml of chymostatin, and 1 μg/ml of pepstatin, and the homogenate was centrifuged at 30,000 $\times$ *g* for 30 min. The total radioactivity, 5% trichloroacetic acid-soluble and insoluble radioactivities of the resulting supernatant were measured separately.

Fluorescent Labeling of Low Molecular Weight Compounds Having Sulfhydryl Groups in the Fat Body—The procedure used was essentially as reported by Fahey *et al.* (*20*). Fat body was washed with buffer A [20 mM *N*-2-hydroxyethylpiperazine-*N*′-3-propanesulfonic acid (HEPPS)/NaOH pH 6.2, 5 mM diethylenetriaminepentaacetic acid, 70 mM KCl, and 70 mM NaCl]. It was then transferred to 0.5 ml of HEPPS/NaOH buffer pH 7.4 (the same as buffer A except for the pH), containing 0.5 mM monobromobimane (mBBr), and kept for 2 min at 4°C with gentle stirring for fluorescence labeling (*21*). Then methanesulfonic acid was added to the mixture to give a final concentration of 200 mM and homogenized. The resulting homogenate was mixed well with an equal volume of 4 M sodium methanesulfonate solution, and the mixture was centrifuged at 8,000 $\times$ *g* for 10 min. The clear supernatant was mixed with an equal volume of methylenechloride with vigorous shaking, and centrifuged at 8,000 $\times$ *g* for 10 min. The aqueous phase was used as a source of fluorescence-labeled low molecular weight substances containing sulfhydryl groups. High performance liquid chromatography (HPLC) of the fluorescence-labeled substances quantitative determination of glutathione were performed as described by Anderson (*22*).

Measurement of the Amount of Glutathione in the Fat Body—Glutathione was measured essentially by the DTNB-GSSG reductase recycling assay method as modified by Griffith (*23*). NADPH, DTNB [5,5′-dithiobis(2-nitrobenzoic acid)], and GSSG (glutathione disulfide) reductase (Sigma) were dissolved in 0.15 M phosphate buffer pH 7.5, containing 6.3 mM EDTA, at a concentration of 0.33 mM, 6 mM, and 10 units/ml, respectively. First, 70 μl of NADPH, 10 μl of DTNB, and 10 μl of sample solution were mixed. Then 10 μl of GSSG reductase solution was added and the yellow color that developed was monitored with time at A_{412} at room temperature. The amount of glutathione (GSH) in the sample was determined from a standard curve obtained with GSSG. By this procedure, total amount of GSH plus GSSG was determined. For determination of the amount of GSSG alone, the sample was treated with 2-vinylpyridine to mask GSH, and then submitted to DTNB-GSSG reductase recycling assay. The fat body was homogenized in 5% 5-sulfosalicylic acid solution and centrifuged at 8,000 $\times$ *g* for 10 min at 4°C, and the resulting supernatant was used as sample solution.

RESULTS

Decrease in the Amount of Sulfhydryl Groups in the Fat Body—A previous *in vitro* study suggested that oxidation of sulfhydryl (SH) groups in the fat body of *Sarcophaga* larvae was necessary for activation of the defense protein genes, because treatment of isolated fat body with 2-mercaptoethanol selectively inhibited the expressions of these defense protein genes (*17*). These genes are known to be activated in the fat body when bacterial suspension is injected into *Sarcophaga* larvae or simply when their body wall is injured. Therefore, we examined if SH groups in the fat body decreased, as detected *in vitro*, under conditions in which these defense protein genes were activated. For this, we harvested fat bodies with time after pricking the body wall of third instar larvae and measured their levels of SH groups that reacted with 2,2′-dithiodipyridine.

As summarized in Table I, the level of SH groups in the fat body decreased about 30% within 30 min after pricking the body wall and this low level of SH groups lasted for at least 3 h. But the level of SH groups was completely restored after 24 h. As we reported previously, *Sarcophaga* lectin mRNA in the fat body becomes detectable 1 h after pricking the larval body wall, reaches a plateau after 3 h, and then decreases gradually, almost disappearing after 24 h (*10*). Thus, we assumed that the content of SH groups in the fat body affects the expression of various defense protein genes both *in vitro* and *in vivo*, and transient decrease in the content of SH groups is prerequisite for the selective activations of these genes.

Identification of the Main Substance Carrying SH Groups in the Fat Body as Glutathione—To determine the

TABLE I. **Decrease in the amount of SH groups in the fat body after injury of the larval body wall of *Sarcophaga*.** The fat body was excised at the indicated times after pricking the body wall of larvae, and its SH group content was determined as described in "MATERIALS AND METHODS."

Time after injury (h)	Amount of SH groups (nmol/mg protein)
0	23.1 ± 1.4^{a}
0.5	$16.5 \pm 1.2^{b,c}$
1	19.3 ± 2.6^{b}
2	$19.3 \pm 2.4^{b,d}$
3	$18.2 \pm 1.4^{b,d}$
24	27.9 ± 4.4^{b}

[a] $n=9$, [b] $n=6$, [c] $p<0.01$, [d] $p<0.05$.

TABLE II. **Subcellular distribution of [^{3}H]NEM-conjugated substances in the fat body.** The fat body was treated with [^{3}H]NEM *in vitro* and fractionated, and the radioactivity in each fraction was determined.

Fractions	cpm/20 μg protein	Percent distribution
30,000 $\times$ *g* ppt.	131 ± 17	0.3
30,000 $\times$ *g* sup.		
TCA-insoluble fraction	$2{,}098 \pm 80$	7.0
TCA-soluble fraction	$27{,}821 \pm 4{,}637^{a}$	92.7

[a] Pretreatment with cold NEM resulted in decrease of this value to 130 ± 42.

amount of substance(s) with SH groups in the fat body, we treated the fat body from normal larvae with [^{3}H]NEM *in vitro* to label SH groups, washed it well, fractionated, and measured the radioactivity of each fraction. As shown in Table II, about 93% of the radioactivity incorporated into the fat body was recovered in the 5% TCA-soluble fraction of the 30,000 $\times g$ supernatant, suggesting that most of the active SH groups are carried by low molecular weight substance(s). This radioactivity is concluded to be due to SH groups conjugated with [^{3}H]NEM, because no significant radioactivity was recovered in this fraction when the fat body was pretreated with 10 mM cold NEM before adding [^{3}H]NEM.

To characterize the low molecular weight substance(s), we treated the fat body with mBBr, a thiol-reactive fluorescent probe, and analyzed the resulting fluorescence-labeled substances by HPLC. As shown in Fig. 1a, a single peak was detected at a retention time of 13 min. This retention time was consistent with that of mBBr-treated GSH, indicating that the low molecular weight substance(s) containing SH groups in the fat body was mainly GSH (Fig. 1c). The amount of GSH in the fat body was found to decrease significantly when the body wall of larvae was injured (Fig. 1b).

Fate of GSH in the Fat Body after Injury of the Larval Body Wall—The peak height of GSH in Fig. 1 suggested that about 20% of the GSH in the fat body disappeared within about 30 min after pricking the larval body wall. To examine the possible conversion of GSH to GSSG under these conditions, we measured the contents of GSSG in the fat body. As summarized in Table III, after pricking the larval body wall, the amount of GSSG in the fat body did not change appreciably, although about 20% of the GSH was reproducibly lost. These results suggest that the loss of

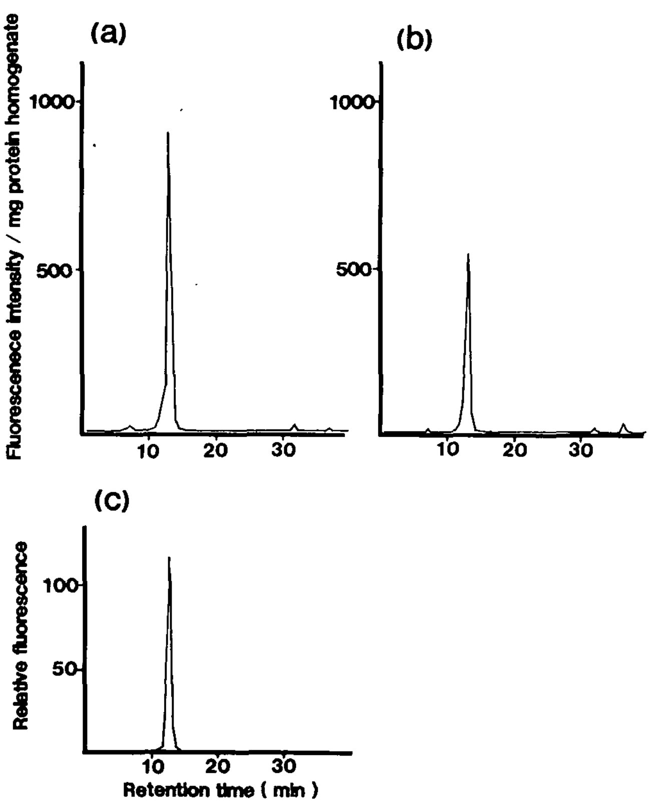

Fig. 1. **HPL chromatogram of mBBr-conjugated low molecular weight compounds.** Fat body was treated with mBBr, homogenized, and subjected to reverse-phase HPLC as described in "MATERIALS AND METHODS." Chromatographic conditions were as follows: Gilson HPLC system with a Synchropak RP-P (C-18) column; isocratic elution with solvent consisting of 14.2% CH_3OH, 0.25% CH_3COOH (pH 3.9). Almost all the mBBr conjugate was eluted under these conditions. Fluorescence was measured in a Hitachi F-3000 spectrophotometer with excitation and emission wave lengths of 385 and 484 nm, respectively. (a) Fat body from control larvae, (b) fat body excised 30 min after injury, (c) mBBr-conjugated GSH (control).

TABLE III. **Amount of GSSG in the fat body.** The fat body was excised 0 (control) and 30 min after pricking the larval body wall, and the amount of GSSG was measured.

Larvae	Amount of GSSG (nmol/mg protein)
Normal	0.14±0.02[a]
Injured	0.15±0.02[a]

[a] $n=7$.

TABLE IV. **Amount of GSH+GSSG in the hemolymph of normal and injured larvae.** Hemolymph samples harvested 0 and 30 min after pricking the larval body wall were deproteinized with 5% sulfosalicylic acid, and subjected to DTNB-GSSG reductase recycling assay.

Larvae	Amount of GSH+GSSG (μM)
Normal	57± 8[a]
Injured	70±10[a]

[a] $n=8$.

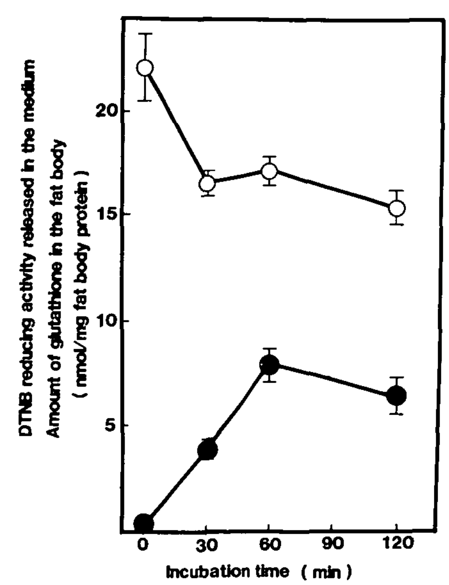

Fig. 2. **Decrease of GSH and release of DTNB-reducing substance from the fat body with time during incubation in buffered insect saline.** The fat body was incubated in buffered insect saline at 27°C. At the indicated times, the content of GSH in the fat body and DTNB-reducing activity in the medium were determined. DTNB-reducing activity was determined by measuring A_{412} after mixing 50 μl of the culture medium and 150 μl of 0.2 mM DTNB solution dissolved in 0.15 M phosphate buffer (pH 7.5) containing 6.3 mM EDTA. The amount of GSH and DTNB-reducing activity were normalized on the base of fat body protein. Points and bars are means and SE of values for 6 samples. (○), content of GSH; (●), DTNB-reducing activity.

GSH is not simply due to its conversion to GSSG.

Therefore we next examined whether about 20% of GSH in the fat body was released into the hemolymph when the larval body wall was injured. For this, we measured the net concentration of GSH and GSSG in the hemolymph by DTNB-GSSG reductase recycling assay, since it was technically difficult to measure the two separately. The content of GSH and GSSG in the hemolymph was much less than that in the fat body, but no significant increase in the net concentration of GSH and GSSG was detected in the hemolymph isolated from injured larvae (Table IV). Total amount of GSH in the fat body was estimated to be about 30 nmol/larva. Release of about 20% into the hemolymph should correspond to about 260 μM assuming that the amount of hemolymph/larva is 30 μl. However, the net concentration of GSH and GSSG in the hemolymph of injured larvae was well below this value, being 70 μM. Thus, part of the GSH in the fat body is probably metabolized *in situ* to an unidentified compound(s) under these conditions.

Consistent with these results, we found that the amount of GSH decreases significantly when the fat body from normal larvae was incubated in the phosphate-buffered insect saline *in vitro*, as shown in Fig. 2. We previously demonstrated that various defense protein genes are activated under these conditions. Concomitant with decrease of GSH, substance that reduced DTNB was found to be released into the medium from the fat body. This substance may not be GSH itself, because NEM did not inactivate this reducing activity and GSH-mBBr was not formed when the medium was treated with mBBr. This substance is very unstable and has not yet been identified. We assume that this DTNB-reducing substance is derived from GSH and the same substance is produced *in vivo* and released into the hemolymph when the larval body wall was injured. However, we could not determine the DTNB-reducing activity in the hemolymph accurately because of the high background level.

DISCUSSION

It is well known that injection of bacteria or injury of the integument of insects induces activation of various defense protein genes in the fat body (*24*). However, nothing is known about the mechanism of this specific gene activation in the fat body. We assume that the intrusion of bacteria or integumental injury creates a certain signal that stimulates the fat body, and selectively activates these defense protein genes.

In this paper, we demonstrated that the GSH level in the fat body decreased significantly when body wall of *Sarcophaga* larvae was injured. Similar decrease in the GSH content was observed when the fat body was incubated in buffered insect saline. Under these conditions, defense protein genes are selectively activated in the fat body. Thus, we think that the decrease of GSH level in the fat body is closely related with the activation of these genes. The basal turnover rate of GSH in the fat body of *Sarcophaga* larvae is very low (unpublished result). Therefore, in *Sarcophaga* there seems to be a special mechanism to reduce the content of GSH in the fat body in response to body injury or bacterial infection, and the same mechanism may be activated when the fat body is simply incubated in buffered insect saline.

An NF-κB-like factor has been suggested to participate in the activation of insect defense protein genes, especially antibacterial protein genes (*14, 16*). In fact, Sun and Faye purified a factor with NF-κB-like properties (CIF) from pupae of *Hyalophora cecropia* (*25*). We also purified a 59-kDa protein that specifically binds to NF-κB-binding motifs of *Sarcophaga* defense protein genes from an embryonic cell line of this insect, and showed that the same protein is present in the fat body (*15*).

Staal *et al.* found that intracellular SH groups, probably due to GSH, decreased significantly when 293.27.2 cells (cell line from human embryonic kidney) were treated with tumor necrosis factor α and/or phorbol 12-myristate 13-acetate, in association with activation of NF-κB (*26*). The decrease of GSH level in their case was at most 25%. They also observed that *N*-acetyl-L-cysteine, a precursor of GSH, prevented this decrease of GSH level and blocked the activation of NF-κB. These findings are consistent with our present results with *Sarcophaga*. Probably, the 59 kDa NF-κB-like protein participates in the activation of defense protein genes in the fat body of *Sarcophaga*, and decrease of the GSH content in the fat body is essential for activation of these genes by the 59 kDa protein. However, nothing is known about why decrease of cellular GSH level induces the activation of the 59 kDa protein.

We found that about 20% of the GSH was lost from the fat body when the larval body wall was injured, and our results suggested that this loss was due to conversion of GSH to an unstable metabolic intermediate having activity to reduce DTNB. Possibly, this metabolic intermediate is needed for activation of the 59 kDa protein. Recently, Galter *et al.* reported that a certain amount of GSSG is required for the activation of NF-κB in human T-cell lineage cells, but that excess GSSG inhibits NF-κB at the level of DNA binding (*27*). In the fat body of *Sarcophaga* larvae, however, we detected no significant increase of GSSG, although the level of GSH decreased. We do not yet know the reason for or the mechanism of decrease of the GSH level in the fat body in response to body injury.

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