We purified a novel antibacterial substance from immunized adult Sarcophaga and determined its molecular structure to be N-β-alanyl-5-S-glutathionyl-3,4-dihydroxyphenylalanine (5-S-GAD). We synthesized 5-S-GAD enzymatically from N-β-alanyl-3,4-dihydroxyphenylalanine (β-Ala-Dopa) and reduced glutathione (GSH). The antibacterial activity of 5-S-GAD was found to be due to its production of H₂O₂. This is a novel antibacterial mechanism as it differs from the mechanisms of known antibacterial peptides. Two possible roles of 5-S-GAD in insect immunity, suppression of bacterial growth and activation of a Rel family transcription factor, are proposed.

It is now well known that dipteran and lepidopteran insects synthesize a battery of defense proteins in response to bacterial challenge or body injury (1–6). These are hemagglutinins and antimicrobial proteins with broad spectra of activity against bacteria and fungi. Usually, the molecular masses of antimicrobial proteins are less than 10 kDa, yet they are derived from independent genes (7, 8). These genes are selectively activated when insects become infected with bacteria or their body wall is injured. The Rel family of transcription factors, such as NF-κB, may participate in the activation of these defense protein genes (9–11).

We have been studying the antibacterial proteins of the flesh fly Sarcophaga peregrina. Cells of this insect induce at least five groups of antibacterial proteins, simultaneously: the sarcotoxins I, II, and III, sapecin, and diptericin (12–16). Each group contains three to five structurally related antibacterial proteins (17, 18). All of these proteins were originally isolated and characterized from larval hemolymph.

Recently, Bulet et al. (19) isolated a novel antibacterial peptide, named drosocin, from an extract of immunized adult Drosophila. This peptide is unique in carrying an O-glycosylated threonine residue, and the saccharide chain (consisting of an N-acetylgalactosamine plus a glucose) was found to be essential for the molecule’s antibacterial activity. We are interested in the structure of the saccharide chain of the Sarcophaga counterpart to drosocin and have tried to isolate it from adult Sarcophaga. So far we have not succeeded in isolating a drosocin-like substance from Sarcophaga, but we have found a novel antimicrobial substance the structure of which we determined to be N-β-alanyl-5-S-glutathionyl-3,4-dihydroxyphenylalanine (5-S-GAD). This paper reports the isolation, structural determination, enzymatic synthesis, and mode of action of 5-S-GAD.

**MATERIALS AND METHODS**

Immunization and Preparation of Crude Extracts of Adult Sarcophaga—Flies were anesthetized by keeping them at 4°C, and were individually pricked with a hypodermic needle that had been dipped in a suspension of Escherichia coli K12594. The insects were kept at 27°C for 24 h and then collected after being anesthetized. The anesthetized flies were homogenized in 10 volumes of 0.1% (v/v) trifluoroacetic acid containing 10 μg/ml aprotinin using a Polytron homogenizer (Kinematica, Luzern). The resulting homogenates were centrifuged for 30 min at 35,000 × g, and the supernatants were filtered through Millipore AA filters (pore size 0.22 μm). The filtrate was used as a crude extract.

Purification of the Antibacterial Substance—The crude extract of immunized flies was loaded onto ODS-AM 120-S50 cartridge (YMC, Kyoto). After washing the cartridge with 0.05% trifluoroacetic acid, the adsorbed material was eluted with 10% (v/v) acetonitrile containing 0.05% trifluoroacetic acid. Known bacterial proteins of Sarcophaga are eluted by 30% acetonitrile and therefore, were not eluted under these conditions. Each fraction was concentrated under vacuum to remove the acetonitrile and trifluoroacetic acid, and the residue was dissolved in water for measurement of the antibacterial activity. Fractions showing antibacterial activity were pooled and applied to a reverse-phase HPLC C18 column (YMC-Pack R&D O-R-ODS-S, 5–5, 120 A, 250 × 4.6 mm; YMC, Kyoto) equilibrated with 0.1% trifluoroacetic acid. After washing the column with 0.1% trifluoroacetic acid, the adsorbed material was eluted using a linear gradient of 0–40% acetonitrile in 0.1% trifluoroacetic acid. Fractions showing antibacterial activity were pooled and subjected to normal-phase HPLC using a TSK gel Carbon-500 column (150 × 4.6 mm; Tosoh, Tokyo) and adsorbed material was eluted using a linear gradient of 0–30% acetonitrile in 0.1% trifluoroacetic acid. At this stage, two peaks containing antibacterial activity were eluted from the column. Fractions containing high antibacterial activity were pooled and again subjected to reverse-phase HPLC using the same type of C18 column. Adsorbed material was eluted using a linear gradient of 0–7.5% acetonitrile in 0.1% trifluoroacetic acid. At this stage, the antibacterial substance eluted as a single symmetrical peak, as detected by UV absorbance at 225 and 280 nm.

**Assay of Antibacterial Activity**—Antibacterial activity was measured in liquid medium using E. coli K12 594 as the indicator bacterium. E. coli was grown in antibiotic medium (Difco). At the exponential phase of growth, cells were collected and suspended in 10 ml phosphate buffer (pH 6.0) containing 130 mM NaCl, 0.2% (w/v) bovine serum albumin, at a density of 2.5 × 10⁸ cells/ml, and the suspension was then diluted 300-fold with antibiotic medium. The sample solution (10 μl) was mixed with 100 μl of the bacterial suspension in each well of a 96-well microtiter plate and incubated at 37°C for 5 h. Then 100 μl of 100 μM

The abbreviations used are: 5-S-GAD, N-β-alanyl-5-S-glutathionyl-3,4-dihydroxyphenylalanine; HPLC, high performance liquid chromatography; GSH, glutathione; HSQC, heteronuclear single-quantum correlation; DQF-COSY, double quantum-filtered correlated spectroscopy; HOHAHA, homonuclear Hartman-Hahn spectroscopy; HMBC, heteronuclear multiple-bond correlation; ESCA, electron spectroscopy chemical analysis.

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phosphate buffer (pH 6.0) was added and the absorbance at 600 nm was measured to assess bacterial growth.

Analysis of 5-S-GAD—Specific rotation was determined in a digital polarimeter (DIP-371, J asco, Tokyo). Amino acid composition was analyzed in an amino acid autoanalyzer (L-8500, Hitachi, Tokyo) after hydrolysis with 6 N HCl containing 4% (v/v) thioglycolic acid at 135 °C for 3 h. UV and IR spectra were recorded using U-3210 spectrophotometer (Hitachi, Tokyo) and a DIP-140 spectrophotometer (J asco, Tokyo), respectively. High resolution-mass spectra were measured using a JMS-SX 102A mass spectrometer (J elol, Tokyo) in the fast atom bombardment mode, using an m-nitrobenzyl alcohol-glycerol matrix with polyethylene glycol as the internal standard. All NMR spectra were recorded using Unity-plus 500 spectrometer (Varian) equipped with a pulse-field gradient system. Element analysis was determined in a Vacuum Generator ESCA (electron spectroscopy for chemical analysis) LAB 5.

Enzymatic Synthesis of 5-S-GAD—The method for 5-S-GAD synthesis was based on the method for synthesizing 5-S-cysteinyl-Dopa (3,4-dihydroxyphenylalanine), as reported by Ito et al. (20). Reduced GSH (121 mg) and β-Ala-Dopa (99 mg) were dissolved in 60 ml of 50 mM phosphate buffer (pH 6.5), and 18 mg of mushroom tyrosinase (EC 1.14.18.1, Sigma) was added. The mixture was kept at room temperature and formation of the conjugate of GSH and β-Ala-Dopa was monitored by the change in the UV spectrum of the reaction mixture. Synthetic 5-S-GAD was purified by reverse-phase HPLC.

RESULTS

Purification of a Novel Antibacterial Substance from Adult Sarcophaga—We tried to isolate the Sarcophaga counterpart to drosocin, which is an O-glycosylated antibacterial peptide of Drosophila, from adult Sarcophaga using a method based on that described by Bulet et al. (19). Most of the known antibacterial proteins of Sarcophaga were removed by the ODS-AM 120-S50 cartridge. A major and a minor peak of antibacterial activity were eluted during the first reverse-phase HPLC separation. We focused on the major peak and tried to purify this substance further. Although we did not detect a Sarcophaga counterpart to drosocin, we were able to purify an unknown substance, with antibacterial activity, to homogeneity by repeating the reverse-phase HPLC procedure (Fig. 1). Results of a typical purification of this substance, extracted from about 10,000 flies, are summarized in Table I.

Molecular Characterization of a Novel Antibacterial Substance—Once the unidentified antibacterial substance had been purified from Sarcophaga, we tried to determine its structure. As the substance was reactive with phenol reagent it was assumed to be a peptide, but we could not determine its N-terminal amino acid residue. Therefore, we analyzed its amino acid composition and found that it contained equimolar Gly, Glu, or Gln, and β-Ala. Besides these amino acids, an unidentified nynhydrin-positive amine was detected. By means of $^1$H NMR and ESCA, this unknown amine was identified as 5-S-cysteinyl-Dopa, which could not be hydrolyzed by 6 N HCl.

The physicochemical properties of this novel antibacterial substance are summarized in Table II. The molecular formula of this substance was determined to be C$_{22}$H$_{31}$N$_3$O$_{11}$S by means of fast atom bombardment high resolution-mass spectra (found: m/z 754.1804; calculated: m/z 754.1819 (M + H)$^+$) in combination with ESCA analysis of sulfur and nitrogen, $^{12}$C NMR, $^1$H NMR, and $^{13}$C HSQC NMR spectrometric analyses. IR absorptions at 3400, 1730, and 1670 cm$^{-1}$ were attributed to hydroxyl, ester, and amide groups, respectively. The maximal absorptions of UV spectra were at 207, 293, and 254 nm.

The NMR spectral data are summarized in Table III. The $^{13}$C and $^1$H NMR spectra indicated that this molecule contains 22 carbons and 30 protons including nine exchangeable protons. The correlations between the chemical shifts of the protons and those of their attached carbons were established by the $^1$H-$^{13}$C HSQC spectrum. The $^1$H spin system of this substance was identified by DQF-COSY and two-dimensional-HOHAHA. On the basis of the results obtained from two-dimensional NMR, we concluded that this substance contains the substructures of Glu, Cys, Gly, β-Ala, and an unknown amino acid residue. The strong NOEs between the Cβ proton resonances of the unknown amino acid residue and the aromatic proton resonances at 6.60 and 6.74 ppm were detected on a NOESY spectrum obtained for the substance.

In order to determine the positions of two aromatic protons and two phenolic protons in the aromatic ring, a $^1$H-$^{13}$C HMBC spectrum was measured. Long-range couplings were observed on the $^1$H-$^{13}$C HMBC spectrum at δH 4.62/δC 36.7; δH 4.62/δC 129; δH 2.74, 2.92/δC 129; δH 2.74, 2.92/δC 125; and δH 2.74, 2.92/δC 116 (Fig. 2). The results obtained from the NOESY and $^1$H-$^{13}$C HMBC spectra clearly indicated that the unknown amino acid residue is Dopa. Furthermore, the $^1$H-$^{15}$N HSQC spectrum indicates that the cross-peaks originating from the Glu and β-Ala residues are due to a secondary amine group. The substructures of this substance, as determined by NMR, are shown in Fig. 2.

The identified structures were then aligned on the basis of the long-range couplings obtained from $^1$H-$^{13}$C HMBC spec-

![Fig. 1. Reverse-phase HPLC profile of a novel antibacterial substance.](image)

**TABLE I**

| Purification step             | Total activity | Total amount | Specific activity | Yield |
|------------------------------|---------------|--------------|------------------|-------|
| Whole body extract           | ND            | ND           | ND               | ND    |
| C18 flash chromatography     | 30542.5       | 95.1         | 321.5            | 100   |
| First-C18 HPLC               | 10189.8       | 8.56         | 1190.4           | 33    |
| Carbon 500 HPLC              | 4532.3        | 3.8          | 1408.5           | 18    |
| Second-C18 HPLC              | 4582.1        | 3.16         | 1450.1           | 15    |

a One unit of antibacterial activity was defined as the amount causing 50% inhibition of bacterial growth relative to the control.
b Total amount of the antibacterial substance was determined by the method of Lowry et al. (13), with bovine serum albumin as the standard.
c ND, not determined.
trum (Fig. 2). Long-range couplings between amide protons and carbonyl carbons were observed as follows: δH 8.61 (Dopa, NH)/δC 127.6 (β-Ala, CO); δH 8.82 (Gly, NH)/δC 173.4 (Cys, CO); δH 8.82 (Cys, NH)/δC 175.1 (Glu, γCO). Long-range coupling between the Cβ protons of the Cys residue and the C5 carbon of the Dopa residue was also observed.

Taking all these data into consideration, we identified this substance as N-β-aryl-5-S-glutathionyl-3,4-dihydroxyphenylalanine, the formula of which is shown in Fig. 3.

Enzymatic Synthesis of 5-S-GAD—As it was difficult to obtain 5-S-GAD in quantity from flies, we tried to synthesize it chemically and enzymatically. First we chemically synthesized β-Ala-Dopa according to the standard procedure, and then we combined β-Ala-Dopa and GSH using tyrosinase, according to the method of Ito et al. (20). The resulting reaction products were separated by reverse-phase HPLC, and the major product was identified as being 5-S-GAD, with a yield of 75%. As shown in Fig. 4, when synthetic 5-S-GAD and authentic 5-S-GAD were mixed and subjected to HPLC, they coeluted as a single peak. We also confirmed the structure of synthetic 5-S-GAD by 1H NMR and fast atom bombardment high resolution-mass spectra.

We compared the antibacterial activity of synthetic 5-S-GAD and authentic 5-S-GAD using E. coli as the target bacterium, and found that the activities of the two substances were the same (Fig. 5). These results indicate that a suitable method for synthesizing 5-S-GAD in quantity has been established.

Novel Antibacterial Mechanism of 5-S-GAD—5-S-GAD was only detected in immunized flies, and not in naive flies, suggesting that it is an inducible defense molecule produced in response to bacterial infection, integumental injury, or both. We found that 5-S-GAD is effective against both Gram-negative and Gram-positive bacteria, with similar IC50 values for E. coli K12 594, Micrococcus luteus FDA 16, and Staphylococcus aureus IFO 12732 of 0.02 to 0.03 μM, under our assay conditions.

We conclude that the antibacterial mechanism of 5-S-GAD is not due to the direct effect of this substance, but to H2O2 produced from it. As shown in Fig. 6, the antibacterial activity of 0.05 μM 5-S-GAD against E. coli was completely abolished by the presence of 100 μg/ml catalase, indicating that H2O2 participates in the antibacterial reaction. Catalase alone had no effect on the growth of E. coli. H2O2 may be generated from the vicinal hydroxyl groups of Dopa, and interferes with bacterial growth. This is a novel antibacterial mechanism for an insect defense molecule.

**DISCUSSION**

We reported the isolation, structural determination, and mode of action of a novel antibacterial substance from Sarcophaga, named 5-S-GAD, which is a conjugate of GSH and β-Ala-Dopa. This insect is known to contain large amounts of β-Ala-Tyr (sarcophagine) (21). Therefore, the β-Ala-Dopa in 5-S-GAD is presumably derived from β-Ala-Tyr. As 5-S-GAD was synthesized from GSH and β-Ala-Dopa via the action of mushroom tyrosinase, which is a phenoloxidase, a similar reaction probably occurs in the insect, which has a potent phenoloxidase.

We purified 5-S-GAD from whole bodies of immunized adult Sarcophaga, so we do not know where 5-S-GAD is synthesized.

**Table II**

Physicochemical properties of a novel antibacterial substance

| Appearance                  | White amorphous solid |
|-----------------------------|------------------------|
| Molecular formula           | C22H31N5O11S           |
| FAB HR-MS (m/2)             | 574.1804(M+)           |
| Calculated                  | 574.1819(M+)           |
| [α]D10 (c 1, H2O)           | −21.8° (c 0.0386, H2O) |
| UV λmax (nm)               | 293(1319), 254(1824), 207(13,996) |
| IR νmax (KBr) cm−1         | 3400, 3100, 3000, 1730, 1670, 1540, 1430, 1200, 1140, 990, 945, 840, 800. |

**Table III**

Summary of 13C, 1H, and 15N NMR spectral data for 5-S-GAD in CD3OH at −55 °C

| Position | δC | δH | δN |
|----------|----|----|----|
| β-Alanine|    |    |    |
| NH2      | 7.81 | 19.0 |    |
| αCH3     | 36.2 | 3.07 |    |
| βCH3     | 32.4 | 2.60 |    |
| CO       | 172.6 |    |    |
| Glutamic acid |      |    |    |
| NH2      | 8.33 | 27.2 |    |
| αCH      | 53.7 | 3.89 |    |
| βCH2     | 26.4 | 2.12,2.16 |    |
| γCH2     | 31.8 | 2.55,2.62 |    |
| μCO      | 173.1 |    |    |
| αCO      | 175.1 |    |    |
| Cystein  |    |    |    |
| NH       | 8.82 | 112.0 |    |
| αCH2     | 49.0 | 4.28 |    |
| βCH2     | 35.6 | 3.14,3.41 |    |
| CO       | 173.4 |    |    |

The proton signal multiplicity and coupling constant (J = Hz) is given in parentheses.
Previously, we reported that the GSH levels in the fat bodies of Sarcophaga larvae transiently decrease by about 30% when the larval body wall is injured (22). This is a rapid response which occurs within 30 min of the injury. Although we have not identified 5-S-GAD in immunized larvae, we assume that the situation is the same between adults and larvae, and that 5-S-GAD is probably synthesized by the fat body, hemocytes, or both. The fat body and hemocytes of Sarcophaga larvae are known to synthesize various defense proteins (23–26). It is not known if 5-S-GAD is secreted into the hemolymph, but in mammalian cells many GSH conjugates are released from the cells via a GS-X pump (27). Thus, it is likely that 5-S-GAD is synthesized in the fat body and/or hemocytes, is released into the hemolymph and suppress bacterial infection.

Mechanism of formation of H$_2$O$_2$ from 5-S-GAD is not clear. Although 5-S-GAD is thought to release H$_2$O$_2$ readily, we cannot exclude the possibility that a specific bacterial oxidase (possibly a terminal oxidase of the respiratory chain) participates in this process in combination with 5-S-GAD.

Finally, we would like to point out the possibility that 5-S-GAD activates a Rel family transcription factor, as well as being an antibacterial substance. As H$_2$O$_2$ is thought to be easily produced from 5-S-GAD in the fat body and/or hemocytes, it is possible that the resulting H$_2$O$_2$ activates a Rel family transcription factor, such as NF-$\kappa$B, in situ resulting in the activation of various antibacterial protein genes. In fact, Sun and Faye (28) have reported that the transcription of immune-protein genes in the fat body of Hyalophora cecropia is augmented by H$_2$O$_2$. In mammals, H$_2$O$_2$ and other reactive oxygen species function as intracellular signals leading to the activation of NF-$\kappa$B (29). Thus, 5-S-GAD may be a key molecule of the insect defense system; in which case, when Sarcophaga was infected with bacteria, 5-S-GAD would be rapidly synthesized and would suppress bacterial growth as the primary response of the insect, while the genes for various antibacterial proteins would subsequently be activated in response to H$_2$O$_2$ produced by 5-S-GAD. This is a novel hypothesis of insect immunity and many more experiments are needed to test it.

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