Microdialysis for the Analysis of Insect Haemolymph

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Microdialysis was used for continuous sampling of biogenic amines from the haemolymph of Spodoptera litura (tobacco cutworm). Six different biogenic amines, including norepinephrine, epinephrine, dopamine, octopamine, tyramine, and serotonin in the microdialyses were simultaneously analyzed by HPLC with a coulometric electrochemical detector. In these samples, dopamine and tyramine existed in relatively high concentrations and octopamine could also be detected. We conclude that the "Insect Microdialysis System" can provide a continuous sampling method from the insect haemolymph without physical stress and consequently increase the sensitivity for detection of changes of the haemolymph biogenic amine level.

In recent years, it has become clear that biogenic amines, such as catecholamines, can act as neurotransmitters, neuromodulators, and circulating neurohormones in insects.1,2 Concentrations of these amines in various organs of the insect have already been studied by many investigators.3-81 On the other hand, in the case of the amines in insect haemolymph, only few studies were done, probably because of the difficulty of analyzing them. Amine concentrations were measured using radio-enzymatic procedure or HPLC, and nowadays the concentrations of haemolymph components are known to be changed by physical and/or chemical stress.9-12 In these studies, insect haemolymph was usually collected from a pinhole made in the insect cuticle. This method cannot be used for the continuous sampling of insect haemolymph and seems not to be adequate for repetitive sampling because of the physical stress and haemolymph leakage caused by the pin holes. In order to understand insect responses to physical and/or chemical stress more precisely, it is necessary to study the delicate changes of amine concentrations in the haemolymph.

In this study, the applicability of microdialysis13 to the continuous sampling from insect haemolymph was investigated. Microdialysis is an advanced and widely used technique for sampling from mammalian tissues. The biogenic amines in microdialyses were analyzed using HPLC with a coulometric electrochemical detector for the high sensitive simultaneous measurement of biogenic amines such as norepinephrine (NE), epinephrine (E), dopamine (DA), octopamine (OA), tyramine (TYRA), and serotonin (5-HT). These amines are typical catecholamines (NE, E, and DA), important amines in insect nervous system (OA and 5-HT), and an intermediate of OA biosynthesis (TYRA).14

Materials and Methods

1. Compounds. Solvents for HPLC analysis were of HPLC grade and all other compounds used in this study were of analytical reagent grade. Octopamine hydrochloride was purchased from the Aldrich Chemical Company, Inc. (Milwaukee, WI, U.S.A.). All other compounds were obtained from Wako Pure Chemical Industries, Inc. (Tokyo, Japan).

2. Insect. The tobacco cutworms, Spodoptera litura, used in this study were reared with an artificial diet, Insecta LF (Nihon Nason, Yokohama, Japan), at 25±1°C under 60-70% relative humidity and 16h daily illumination. In this study 1-2 day-old 5th instar S. litura larvae were used.

3. Insect saline. The insect saline was prepared as described by Davenport and Wright (1985).15 It contained 7.1 mm CaCl₂, 22.0 mm Na₂HPO₄, 13.5 mm MgSO₄, 0.1 mm CaCl₂, 6H₂O, 29.5 mm KCl, and 29.3 mm glucose, and was finally adjusted to pH 6.8 by addition of aqueous KH₂PO₄.

4. HPLC analysis. Concentrations of biogenic amines were measured by HPLC with an electrochemical detector. Identification of compounds was done by cochromatography with known standards and by the electrochemical characteristics of the compounds. The internal standard was not used in this study, because the retention time and the intensity of standard samples were reproducible before and after the analyses of microdialysate samples. The chromatography was done on a 200×4 mm i.d. Wako-50% 218AR column (Wako) protected with a 10×4 mm i.d. Wako-50% 218AR guard column (Wako) at 45°C by a CTO-10A column oven (Shimadzu, Kyoto, Japan). The mobile phase comprised 75% HPLC-buffer (pH 2.8), 15% acetonitrile, and 10% methanol containing 0.07% SDS. The HPLC-buffer was prepared from 75 mm sodium dihydrogenphosphate and 20 mm trichloroacetic acid and was filtered through a 0.20 mm cellulose nitrate membrane filter (Toyo Roshi, Tokyo, Japan). The mobile phase was degassed by ultrasonic waves under vacuum pressure and was pumped by a LC-10AD pump (Shimadzu) with a high sensitivity filter unit (Shimadzu) at a flow rate of 1.0 ml/min. Samples were injected into the column using a 7125 syringe loading injector (Rheodyne, Cotati, CA, U.S.A.) fitted with a 200 μl sample loop. Biogenic amines were detected by a coulometric electrochemical detector, Coulonics (ESA, Bedford, MA, U.S.A.) with a 5020 guard cell and a 5010 dual electrode analytical cell. The guard cell was set at +100 mV and placed before the analytical cell to screen out electrochemical impurities from the analytical cell. The first electrode of the analytical cell was set at +350 mV to detect NE, E, DA, and 5-HT, and the second one was set at +650 mV to detect OA and TYRA.

5. Sample purification. The disposable extraction column, Bond Elut
CBA 100 mg. (Analytichem International, Harbor City, CA, U.S.A.) was washed with methanol and 1% perchloric acid (PCA) before use. The column was conditioned with 10 mM sodium phosphate buffer (pH 7.0) and 50–100 μl of the sample solution was put on it with 0.5 ml of the buffer. The fluid was drawn through the column using low vacuum pressure and the column was then rinsed sequentially with 0.5 ml of the buffer and 0.2 ml of 0.1% PCA. Biogenic amines were eluted from the column with 0.2 ml of 1% PCA. The appropriate portion of eluate was analyzed by HPLC.

6. Microdialysis

6.1 General method. A 1–2 day-old 5th instar S. litura larvae was immobilized on a styrene foam board by taping tape C-12F (Nichiban, Tokyo, Japan) at 23 ± 1°C under 50–60% relative humidity and constant illumination. A microdialysis probe CMA/12 (Carnegie Medicin, Stockholm, Sweden) was used in this study. This probe has a needle-like stainless steel shaft (14 × 0.64 mm o.d.) with a dialysis membrane (4 × 0.5 mm o.d.). The membrane is made by a polycarbonate and its cut-off molecular mass is 20,000 daltons. The probe also has an inlet and an outlet port connected to a pump and a sample cup by Teflon lines (200 × 0.12 mm i.d.), respectively. Microdialysis is done by circulating the solution from the inlet port of the probe, dialyzing at the membrane region and then collecting the dialyzed sample (so-called microdialysate) from the outlet port. In this study, sampling was done as follows. The probe was inserted ahead from the side of the insect abdomen using a guide cannula (Carnegie Medicin), which was fixed to the insect cuticle by an adhesive agent, Alanolpha-201 (Toa, Tokyo, Japan) and Scotch tape (Sumitomo 3M, Tokyo, Japan). The twice-diluted insect saline was pumped at a flow rate of 4 μl/min using a syringe pump STC-521 (Telumo, Tokyo, Japan). After 5 min of flow, the eluate was collected every 15 min for 2 h into the ice-cold polypropylene micro tube and stored below −20°C until analysis.

6.2 Application of physical stress. In order to estimate the physical stress caused by inserting the microdialysis probe into the insect, the changes in the biogenic amine concentrations in the stressed insect were also investigated. Sampling was done in a similar manner as described above. After 1 h from the beginning of the sampling, physical stress was applied by inserting a stainless steel needle at the opposite side of the insect abdomen to where the microdialysis probe was inserted.

6.3 Recovery test of microdialysis. A microdialysis probe CMA/12 was dipped in 2 ml of insect saline containing 1 μg of six biogenic amines (NE, E, DA, OA, TYRA, and 5-HT). These amines were sampled by circulating the twice-diluted insect saline at a flow rate of 4 μl/min as described above for 30 min. The eluate was directly analyzed by HPLC and concentrations of these amines were calculated. This recovery test was repeated three times and recovery rates in this condition were calculated.

Results and Discussion

1. HPLC analysis

With the Coulloch II detector, a guard cell is usually placed before the injector to remove electrochemical impurities from the HPLC mobile phase and thus decrease the background current of the analytical cell. NE and E in the microdialysates from S. litura haemolymph were hardly to be analyzed by such an HPLC system configuration, because of the interference by the high level of electrochemical impurities in the haemolymph. In this study, the guard cell was placed between the column and the analytical cell and the analyses were done under the screen mode using three electrodes. Six biogenic amines could be well analyzed by setting the guard cell and the analytical cell (first and second electrodes) at +100, +350, and +650 mV, respectively, as shown in Fig. 1. In this detector condition, impurities that have the highest electroactivities were screened out by the guard cell and relatively electroactive amines (NE, E, DA, and 5-HT) could be detected at the first electrode of the analytical cell and then electrically less active amines (OA and TYRA) were detected at the second one. The detection limit of six biogenic amines was approximately 50 fmol/injection.

2. Sample purification

It is necessary to purify biogenic amines in microdialysates of S. litura haemolymph before the HPLC analysis. For catecholamines, the adsorption on alumina or on boric acid gel is the most frequently used method because of their unique selectivity towards catechol groups. This method cannot be applied to monophenolic compounds (OA and TYRA) or a hydroxyindole compound (5-HT), so that Bond Elut CBA, a cation exchange resin, was used in this study. Using this clean-up method, six biogenic amines were purified based on their amino groups. Recovery rates were good for NE, E, DA, OA, and TYRA, and that of 5-HT was not so good but could be analyzed as shown in Table I.

<table>
<thead>
<tr>
<th>Run No.</th>
<th>NE</th>
<th>E</th>
<th>OA</th>
<th>DA</th>
<th>TYRA</th>
<th>5-HT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run-1</td>
<td>94.2</td>
<td>97.6</td>
<td>99.5</td>
<td>90.3</td>
<td>103.0</td>
<td>69.8</td>
</tr>
<tr>
<td>Run-2</td>
<td>98.3</td>
<td>92.3</td>
<td>97.6</td>
<td>84.4</td>
<td>98.3</td>
<td>59.8</td>
</tr>
<tr>
<td>Run-3</td>
<td>95.0</td>
<td>90.7</td>
<td>98.6</td>
<td>84.0</td>
<td>104.6</td>
<td>68.7</td>
</tr>
</tbody>
</table>

Average: 95.8% 93.5% 98.5% 86.2% 102.0% 66.1%
S.D.: 1.81 2.97 0.76 2.86 2.66 4.45

3. Microdialysis

Recovery rates of biogenic amines for microdialysis were good with small standard deviations as summarized in Table II. This sampling method being basically by dialysis, the recovery rate was improved by circulating the diluted solution rather than the insect saline itself. In the case of OA and DA, for example, their recovery rates by using the twice-diluted saline were better (13.0% and 10.0%, respectively) than those by using the insect saline itself (6.8% and 7.0%, respectively). Concerning the interference by impurities and the avoidance of haemolymph dilution, the twice-diluted insect saline was used.

Microdialysis is widely used for sampling from mammalian tissues; however, this is the first report on the
Table II. Recovery Rates for Microdialysis

<table>
<thead>
<tr>
<th>Run No.</th>
<th>NE</th>
<th>E</th>
<th>OA</th>
<th>DA</th>
<th>TYRA</th>
<th>5-HT</th>
</tr>
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<tbody>
<tr>
<td>Run-1</td>
<td>9.62</td>
<td>8.28</td>
<td>12.39</td>
<td>7.72</td>
<td>12.57</td>
<td>12.97</td>
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<td>Run-2</td>
<td>10.44</td>
<td>9.91</td>
<td>14.52</td>
<td>13.06</td>
<td>15.34</td>
<td>14.12</td>
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<tr>
<td>Run-3</td>
<td>9.19</td>
<td>7.84</td>
<td>12.11</td>
<td>9.12</td>
<td>13.72</td>
<td>15.05</td>
</tr>
<tr>
<td>Average</td>
<td>9.75</td>
<td>8.68</td>
<td>13.00</td>
<td>9.97</td>
<td>13.88</td>
<td>14.05</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.52</td>
<td>0.89</td>
<td>1.07</td>
<td>2.26</td>
<td>1.14</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Fig. 2. Examples of Changes in Biogenic Amine Concentrations in Insect Haemolymph.

Details were described in Materials and Methods. ●, dopamine; ○, tyramine; ▲, octopamine.

The application of microdialysis to the insect haemolymph as far as we know. In this study, the 1—2 day-old 5th instar S. litura larvae were used because of their large body sizes, sufficient for inserting a microdialysis probe (4 × 0.5 mm o.d.). The leakage of haemolymph from the pin hole made on the insect cuticle was negligible if the probe was fixed by the adhesive agent. The effect of physical stress applied by inserting a needle seemed to be negligible in this assay system (data not shown). Therefore, the effect of physical stress caused by inserting the microdialysis probe, which has a similar size to the needle, was also thought to be small. The insects were confirmed to be alive for at least 6 h after injection of the microdialysis probe. The examples for changes in biogenic amine concentrations in the normal insect haemolymph are shown in Fig. 2A and 2B. Generally, in the case of the 1—2 day old 5th instar S. litura larvae, DA and TYRA were at relatively high concentration and OA could also be detected. Comparing Fig. 2A with Fig. 2B, it was known that the great individual differences existed in the amine concentrations in the insect haemolymph. Such individual differences were observed previously. It makes it difficult to analyze the data precisely and masks delicate responses because of the large standard deviations. In this regard, the microdialysis, which makes it possible to sample the haemolymph continuously, is the only way to study the delicate changes of the haemolymph components in a single insect and is useful for studying the effects of various stresses on the levels of haemolymph components.

Now we are investigating the effects of chemical stress on the levels of biogenic amines in S. litura haemolymph using this analysis system.

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References