Determination of Polyamines by Radioimmunoassay with Antiserum Against o-Phthalaldehydic Spermine

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SUMMARY Antisera against spermine were obtained by immunization of rabbits with o-phthalaldehydic spermine conjugated to bovine serum albumin. Characterization of the antiserum and radioimmunoassay of polyamines were described. Polyamine can be determined after reaction with o-phthalaldehyde. As little as 40 and 30 pmol of respective spermine and spermidine were detected by this assay.

Introduction

Although polyamines have been known to play an important role in various metabolic regulations in vivo, such as involvement of cell-multiplication, stability of nucleic acid, acceleration of protein and nucleic acid syntheses and regulation of various enzyme activities, problems with their analysis make it difficult to elucidate the more apparent in vivo functions or roles of polyamines.

In order to obtain antiserum against spermine, we have followed the method of Bartos et al. in which the hapten had been conjugated to thyroglobulin with the carbodiimide procedure, but we did not succeed in production of antispermine antibody.

Since it was thought that spermine has less antigenicity for its short straight-chain structure, we attempted modification of spermine molecule to enhance its antigenicity. o-Phthalaldehydic spermine was antigenic and we obtained its corresponding antiserum by repeated immunization of rabbits. The antibody obtained made possible the establishment of a radioimmunoassay of polyamine.

Materials and Methods

Preparation of antigen

o-Phthalaldehydic spermine (Sp-OPT) was obtained by the method of Kremzner with a modification as shown in Fig. 1. Spermine tetrahydrochloride (50 mg) was dissolved in 40 ml of 0.5N NaOH. To this solution were added 10 ml of 1% OPT
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Fig. 1 Synthesis of \( o^-\text{phthalaldehydic spermine} \) conjugated to bovine serum albumin (Sp-OPT-BSA) by diazo-coupling.

| BSA-NH\(_2\) + CICOCH\(_2\)-NO\(_2\) | H\(_2\)N-(CH\(_2\)_\(_5\)-NH-(CH\(_2\)_\(_4\)-NH-(CH\(_2\)_\(_3\)-NH\(_2\) | + CHO  
| BSA-NHCO-CH\(_2\)-NO\(_2\) |  
| BSA-NHCO-CH\(_2\)-NH\(_2\) | + HNO\(_2\)  
| BSA-NHCO-CH\(_2\)-N=N-N-(CH\(_2\)_\(_5\)-NH-(CH\(_2\)_\(_4\)-NH-(CH\(_2\)_\(_3\)-N |  

methanolic solution, stirred with a magnetic stirrer at room temperature for 1 h. The mixture was acidified with HCl and unreacted OPT was extracted with diethyl ether. The aqueous phase was then alkalized with NaOH and the precipitate was collected by filtration.

Fifty mg of bovine serum albumin (BSA) were dissolved in 4 ml of 0.02 N NaOH and cooled in an ice bath. To this BSA solution, 2.0 ml of \( p^-\text{nitrobenzoyl chloride} \) \( (p^-\text{NBC}) \) ether solution (containing 2.7, 5.5 or 8.2 mg each as \( p^-\text{NBC} \)) were added under stirring dropwise and stirred for 30 min, maintaining the pH between 8.5 and 9.0 with 0.02 N NaOH solution. \( p^-\text{NBC} \) was then added to BSA at three kinds of molar ratio, i.e., 20, 40 and 60. The mixture was centrifuged and the aqueous phase was dialyzed against running water for 2 days. The reactant was dissolved in 4 ml of 0.1 M Na\(_2\)SO\(_4\) solution containing 0.5 M NaHCO\(_3\) (pH 8.5) and incubated at 37\( ^\circ\)C for 5 h to make the amination. The mixture was dialyzed against running water for 2 days. The pH of the dialysate was adjusted to 1.5 with 1 N HCl, and after 4.14 mg of NaNO\(_2\) in 1 ml of water were added dropwise to the dialysate and mixed at 0-4\( ^\circ\)C for 10 min, the excess HNO\(_2\) was then neutralized with 1% ammonium sulfamate aqueous solution.

Sp-OPT \((13 \text{ mg})\) was dissolved in 1 ml of dimethylformamide and further 10 ml of 0.1 M borate buffer (pH 9.0) were added. The above diazotized BSA-conjugate solution was added dropwise to this solution at 0-4\( ^\circ\)C and mixed overnight, dialyzed against running water for 4 days and the dialysate was lyophilized as immunogen.

For the estimation of the amounts of \( o^-\text{phthalaldehydic spermine} \) coupled to the carrier protein, tritium-labelled spermine was added in the first reaction step of Sp-OPT synthesis, and the radioactivity of the dialysate was measured, the degree of conjugation being calculated on each dialysate to be 27, 40 and 50 to the corresponding ratio of \( p^-\text{NBC} \).

**Immunization**

A 1.0 ml volume of Sp-OPT-BSA solution in saline, which contains 1 mg protein, was emulsified with an equal volume of complete Freund’s adjuvant. Domestic male albino rabbits, weighing 2.5-3.0 kg, were injected subcutaneously at multiple sites in the back and foot pad. Three rabbits in each group according to the molar ratio of the hapten to BSA
received 2 ml of the emulsion twice during the first month and then once every 3 weeks for 5 months. Blood samples were collected from the vein of the rabbit ear 7-10 days after each injection for the test of binding activity of the antiserum. Ten days after the last booster injection, the rabbit was bled from the carotid artery under no anaesthesia, and the blood allowed to clot. The serum was separated by centrifugation and inactivated at 56°C for 30 min. The serum obtained was kept at -20°C until used as the source of antibody.

Radioimmunoassay procedure

For the dilution of antiserum and for the preparation of labelled [³H] Sp-OPT and of cold ligand solutions 0.05 M Tris-HCl buffer (pH 7.4) was used. Each assay tube contained the following components: 0.2 ml of [³H] Sp-OPT (about 5000 dpm, 104 fmol), 0.2 ml of cold ligands and 0.1 ml of diluted antiserum (1:20) which binds about 50% of the constant amount of the labelled Sp-OPT. The solution was then mixed with a Vortex mixer and incubated at room temperature for 2 h. After the addition of 0.5 ml of saturated ammonium sulfate solution to the reaction mixture, the precipitate containing immunocomplex was immediately collected by centrifugation at 3000 rpm for 15 min at room temperature. The precipitate obtained was then washed twice with 0.5 ml of 50% saturated ammonium sulfate solution and then dissolved in 1.0 ml of distilled water; 0.5 ml of this solution was transferred to 10 ml of dioxane scintillator and the radioactivity was measured in a liquid scintillation spectrometer.

Chemicals

[³H] Spermine tetrahydrochloride (specific activity 21.7 Ci/mmole) was purchased from New England Nuclear. Polyamines listed in Table I were purchased from Nakarai Chemicals, L-arginine and L-lysine from Nippon Rikagakuyakuhin and L-(+)-ornithine from Aldrich. Agmatine, OPT and other common reagents were purchased from Wako Pure Chemical Industries. Polyamines and their related compounds were reacted with OPT for cross-reaction tests of anti-Sp-OPT antiserum as described in the method of Sp-OPT preparation.

Results and Discussion

The proposed chemical structure of Sp-OPT illustrated in Fig. 1 was estimated by formulae shown in references 1-7. Five months after the initial injection, the blood serum obtained from the immunized rabbit showed an increased binding activity to Sp-OPT. Each 1:20 diluted antiserum from 3 rabbits of 3 different groups did not differ significantly in binding activity against the hapten, that is from 20 to 50% of [³H] Sp-OPT (about 5000 dpm), of which

![Fig. 2 Displacement of [³H] o-phthalaldehydeic spermine (Sp-OPT) bound to the 1:20 diluted anti-Sp-OPT-BSA serum by unlabelled Sp-OPT. Normal rabbit serum gave several to 10% binding of the 5000 dpm of labelled Sp-OPT. The values plotted were not corrected for the background.](image-url)
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Table I Cross-reactivity of anti o-phthalaldehydic spermine (Sp-OPT)

<table>
<thead>
<tr>
<th>Ligands</th>
<th>Cross-reactivity (%)</th>
</tr>
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<tbody>
<tr>
<td>Spermine-OPT</td>
<td>100</td>
</tr>
<tr>
<td>Spermidine-OPT</td>
<td>133</td>
</tr>
<tr>
<td>Cadaverine-OPT</td>
<td>18.2</td>
</tr>
<tr>
<td>Putrescine-OPT</td>
<td>25</td>
</tr>
<tr>
<td>Agmatine-OPT</td>
<td>8.9</td>
</tr>
<tr>
<td>Arginine-OPT</td>
<td>0.45</td>
</tr>
<tr>
<td>Lysine-OPT</td>
<td>8.9</td>
</tr>
<tr>
<td>Ornithine-OPT</td>
<td>7.4</td>
</tr>
<tr>
<td>OPT</td>
<td>(&lt;0.002)</td>
</tr>
<tr>
<td>Spermine</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>Spermidine</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>Cadaverine</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>Putrescine</td>
<td>(&lt;0.001)</td>
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</table>

The antiserum binds well to polyamine-OPT's, slightly to basic amino acid-OPTs and negligibly to both OPT and polyamines. Not only unlabelled Sp-OPT but spermidine-OPT inhibits \(^{3}H\) Sp-OPT binding to the antiserum; the binding affinity of the latter compound for the antibody was higher than that of the former one (Table I). These findings indicate the existence of an antibody possessing a site which binds to the structure "OPT-N-(CH\(_2\))\(_3\)-NH-(CH\(_2\))\(_4\)-N-" shared in both Sp-OPT and spermidine-OPT molecules. Accordingly, one molecule of spermidine-OPT is thought to be occupied by one molecule of the antibody. On the other hand, one molecule of Sp-OPT is assumed to be occupied by at least more than one molecule of the antibody, namely the above cited portion of the Sp-OPT ought to bind one molecule of the antibody and the residual portion of the Sp-OPT, "OPT-N-(CH\(_2\))\(_3\)-", would be occupied in part by the antibody, because of the fact that the "OPT-N-(CH\(_2\))\(_3\)-" portion is also suggested to be partially occupied by the antibody shown by the date of the cross-reactivities of cadaverine-and putrescine-OPT. From these results the amount of \(^{3}H\) Sp-OPT occupied by constant molecules of the antibody is to be larger in a tube containing unlabelled spermidine-OPT than in one containing unlabelled Sp-OPT, appearing to explain the fact that the 133\% cross-reaction of spermidine-OPT was observed.

The radioimmunoassay using antiserum against Sp-OPT-BSA described is relatively sensitive as 40 pmol of spermine and smaller amounts of spermidine than of spermine can be determined. This method would be advantageous for measurement of each polyamine in biological materials after separation by other methods such as
high-performance liquid chromatography\textsuperscript{10}. The radioimmunoassay is more sensitive than fluorometry of spermine and spermidine using monoamine oxidase\textsuperscript{11}, so that the method can be utilized, for instance, by detecting semen containing large amounts of spermine from biological samples in sexual offence.

\textbf{Acknowledgement}

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\textbf{References}