ISOPEROXIDASE LABELED ANTIBODIES: EFFECTS ON SPECIFIC AND NON-SPECIFIC REACTIONS*

AKIRA KAWAOI

The First Department of Pathology, Nihon University School of Medicine, Tokyo, 173

The efficiency of isoperoxidase labeled antibodies was examined using the antigen coupled Sepharose 4B system as well as the tissue staining technique. Four isoenzymes including acidic and neutral fractions proved to be useful as marker enzymes for immune reaction. Some differences in the specific and non-specific reactivities of the isoenzyme-antibody conjugates were also demonstrated.

Non-specific staining in the immuno-enzyme technique is caused by many factors, among which the nature of the enzyme-immunoglobulin conjugates is thought to be important. There are several isozymes of horseradish peroxidase which have different amino acid composition, isoelectric point, carbohydrate content and so forth (2, 3).

The peroxidase-immunoglobulin complexes which are used in immuno-enzyme staining are expected to differ from isoenzyme to isoenzyme in efficiency for staining. In the present study four isoenzymes of horseradish peroxidase were coupled to antibody and their suitability for immuno-enzyme staining was examined.

MATERIALS AND METHODS

Isoperoxidases

The four isoperoxidases isolated from crude horseradish peroxidase were the products of Toyobo Co., Ltd. The isolation pattern on the SP-Sephadex C-50 column chromatography is shown in Fig. 1. Four fractions were isolated and designated as P-1, P-2, P-3 and P-4. These fractions are characterized in Table 1.

Conjugation of the isoperoxidases with anti-rabbit gammaglobulin goat gammaglobulin

The conjugation procedure followed the method described by Nakane and Kawaoi (1). The molar ratio of the enzyme-gammaglobulin conjugates (E/G ratio) was estimated according to a modified version of the formula of Yamashita et al. (4).

Preparation of the rabbit gammaglobulin or porcine liver powder coupled Sepharose 4B.

Five grams of CNBr-activated Sepharose 4B (Pharmacia) dissolved in 50 ml

* Supported in part by a Grant-in-Aid for Cancer Research (101003) from the Ministry of Education, Science and Culture, Japan.
of 0.1 M Atkins-Pantin buffer (pH 8.5) with 0.5 M NaCl was mixed with 20 ml of rabbit gammaglobulin (RGG), 10.7 mg/ml in the same buffer, and incubated for 3 hr at room temperature. The RGG coupled Sepharose 4B (RGG-S) was washed, further incubated with 1.0 M ethanolamine (pH 8.0) for 2 hr at room temperature, and finally suspended in 20 ml of 0.01 M phosphate buffered saline (pH 7.2) PBS.

Porcine liver acetone powder (Sigma) was coupled to Sepharose 4B in the same manner (LAP-S).

The non-coupled Sepharose 4B (N-S) was prepared according to same produce except that no protein was added.

**Reaction procedure of isoperoxidase labeled anti-RGG with the coupled or non-coupled Sepharose 4B.**

0.5 ml of the coupled or non-coupled Sepharose 4B was diluted to 20 times the original volume by Sephadex G-200 (Pharmacia), suspended in 0.4 ml PBS and incubated with 0.1 ml of the isoperoxidase labeled anti-RGG (isoHRP-anti-RGG) which had been appropriately diluted by 1% bovine serum albumin (BSA) in BBS. It was then incubated for 2 hr and 30 min at room temperature, followed by centrifugation and washing. The enzyme activities of the isoHRP-anti-RGG and the isoHRP-anti-RGG reacted Sepharose were detected by

**TABLE 1. Isoperoxidase characteristics (Toyobo)**

<table>
<thead>
<tr>
<th></th>
<th>RZ</th>
<th>PU/mg</th>
<th>Fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-1</td>
<td>0.98</td>
<td>152</td>
<td>acidic</td>
</tr>
<tr>
<td>P-2</td>
<td>2.33</td>
<td>232</td>
<td>neutral</td>
</tr>
<tr>
<td>P-3</td>
<td>2.79</td>
<td>306</td>
<td>neutral</td>
</tr>
<tr>
<td>P-4</td>
<td>2.68</td>
<td>277</td>
<td>neutral</td>
</tr>
</tbody>
</table>
0.1% o-aminophenol solution with 0.08% H₂O₂ in 0.1 M phosphate buffer (pH 6.0) at O. D. 470 nm.

Staining of the tissue sections

In order to test the immunological reactivity of the isoHRP-anti-RGG on the tissue sections, the paraformaldehyde fixed paraffin sections of the pituitary middle lobes of rat were reacted with MSH cross-reacting anti-ACTH rabbit serum diluted 1:10 with PBS, and then with the isoHRP-anti-RGG. The enzyme activity of the conjugates was detected by 3,3'-diaminobenzidine solution with 0.005% H₂O₂ (Karnovsky).

RESULTS

1. Sephadex G-200 column chromatography pattern of the isoHRP-anti-RGG.

   The isoHRP-anti-RGG were run through the Sephadex G-200 column. Their elution patterns are shown in Fig. 2. Each fraction was gathered into 3 main fractions, Nos. 1, 2 and 3 as shown in Fig. 2. Fractions 1 and 2 contain the iso HRP-anti-RGG and fraction 3 is the unconjugated HRP. These fractions were concentrated to the original volume and stored at 4°C.

2. The molar ratio of the HRP-gammaglobulin conjugates (E/G ratio).

   The calculated E/G ratios of fractions 1 and 2 are shown in Table 2.

3. Specific and non-specific reactions of the conjugates with the RGG-S, LAP-S and N-S.

   The percentage of binding in each fraction of the conjugates is shown in Fig. 3.

![Fig. 2. The elution patterns of the isoHRP-anti-RGG. ---: OD 280 nm. ---: OD 403 nm.](image-url)
4. The specific staining of each conjugate by fractions 1 and 2 is estimated in Table 3.

The results of the staining of the isoperoxidase P-2 conjugate with fractions 1 and 2 are shown in Figs. 4 and 5, respectively. Fraction 1 revealed satisfying stainability, whereas fraction 2 showed an almost completely negative reaction with some non-specific granular depositions.

<table>
<thead>
<tr>
<th>Fr.</th>
<th>P-1</th>
<th>P-2</th>
<th>P-3</th>
<th>P-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.0</td>
<td>3.4</td>
<td>3.2</td>
<td>3.1</td>
</tr>
<tr>
<td>2</td>
<td>4.3</td>
<td>3.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

**Table 2. E/G molar ratio of the isoHRP-anti-RGG**

**Table 3. Specific staining on tissue sections**

<table>
<thead>
<tr>
<th>Fr.</th>
<th>P-1</th>
<th>P-2</th>
<th>P-3</th>
<th>P-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+ ~ ++</td>
</tr>
<tr>
<td>2</td>
<td>+ ~ ++</td>
<td>- ~ ±</td>
<td>++</td>
<td>-</td>
</tr>
</tbody>
</table>

**Fig. 3.** The binding of the isoperoxidase conjugates to Sepharose 4B uncoated or coated with RGG or LAP.
DISCUSSION AND CONCLUSION

The present study showed that the isoperoxidases P-1, 2, 3 and 4 are all utilizable for the labeling of immunoglobulin, with there being some differences in the intensity and specificity of the reaction with the insolublized Sepharose 4B coupled or tissue antigen. The reactivity also depends on the fractions of the same isoenzyme conjugate.
The results of tissue staining with each fraction of the isoperoxidase conjugates were well correlated to those obtained from the antigen coupled Sepharose system. Fraction 1 of the P-2 and P-4 conjugates and fraction 2 of the P-1 and P-3 proved to be highly efficient in that the specificity of the binding was excellent and the non-specific reactivity was weak. On the other hand, fraction 2 of the P-2 and P-4 conjugates was demonstrated to be very poor in specific reactivity and fairly high in non-specific binding.

The calculated E/G ratios of the conjugates didn’t seem to significantly influence the efficiency of the conjugates in specific and non-specific reaction.

The antigen coupled Sepharose 4B system was found to be useful in testing the reactivity of the labeled antibody and in establishing conditions for immunohistochemical reactions.

ACKNOWLEDGEMENTS

The isoperoxidases were kindly offered by Toyobo Co., Ltd. The author is indebted to Mr. M. Otaki and Miss H. Satoh for technical assistance and to Miss H. Tajima for preparing the manuscript.

REFERENCES