〔Original〕

Production of Hybridoma Secreting Specific Antibody against Horseradish Peroxidase Isozyme

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Abstract: The specific antibody against horseradish peroxidase isozyme was prepared by a cell fusion technique between peroxidase-immunized mouse splenocyte and myeloma, P3-X63-U1 AZG^R. The prepared antibody could specifically bind to Type VIII isozyme and repress its activity. In contrast, the conventional antiserum could not differentiate the specific antigen sites of isozymes.

Key words: hybridoma, specific antibody, horseradish peroxidase isozyme.

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Introduction

Horseradish peroxidase has been known as a useful marker enzyme to follow the cell lineage or to detect the various antigens by the enzyme-linked antibody. The localization of this enzyme is usually detected by the bentidine-reaction or its antibody. However, it is sometimes difficult to determine the accurate position of this enzyme or corresponding antigen because of the diffuse and smear staining pattern. This may be caused by the heterogenous nature of the preparation of isozymes and antibodies.

In this paper, in order to develop a more selective and accurate method, the authors prepared successfully a specific antibody against Type VIII isozyme by hybridoma between enzyme-immunized mouse splenocyte and a myeloma cell. Then, the secreted antibodies were partially characterized.

Materials and Methods

Chemicals

Horseradish peroxidases were purchased from Sigma; crude enzyme (Type I, Lot No. 25C-9520), partially purified enzyme (Type II, Lot No. 120F-9700), acidic isozymes (Type VII, Lot No. 107C-9720, and Type VIII, Lot No. 110F-9730) and basic isozyme (Type IX, Lot No. 44C-9610). Goat anti-peroxidase serum (Miles Yeda, Lot No. 25C-9520), IgG

Table 1. Screening for the antibody-producing hybridoma cell line by PHA test

Dilution (2 ⁿ -fold)	Cell lines										
	B11	D5	E6	F5	F7	G11	B7	G6	S1	S2	C
1	—	—	—	—	—	—	+	+	+	+	—
2	—	—	—	—	—	—	+	+	+	+	—
3	—	—	—	—	—	—	+	+	+	+	—
4	—	—	—	—	—	—	+	+	+	+	—
5	—	—	—	—	—	—	—	+	+	+	—
6	—	—	—	—	—	—	—	—	—	+	—
7	—	—	—	—	—	—	—	—	—	+	—
8	—	—	—	—	—	—	—	—	—	+	—
9	—	—	—	—	—	—	—	—	—	+	—
10	—	—	—	—	—	—	—	—	—	—	—

S1 and S2 are 10 fold-diluted goat anti-peroxidase serum and prepared mouse antiperoxidase serum, which was described in materials and methods, respectively. C was incubated with 3 μ g of non-specific IgG as the control experiment.

Table 2. Effects of antibodies on the peroxidase isozymes

	Horseradish peroxidase activity (% of control activity*)		
	Isozyme type		
	VII	VIII	IV
(a) Anti-peroxidase serum			
Goat	89	89	90
Mouse	93	93	89
(b) Hybridoma supernatant			
F7	100	100	102
G6	101	82	100
B7	101	91	101

2 μ g of goat antiserum antibody and 5 μ l of original mouse serum were used in each assay, respectively. Control enzyme activity at 436 nm was about 0.70 (0.65 – 0.73) and the absorbance of substrate solution was less than 0.02.

* Presented value was the average activity of duplicate assays.

fraction sheep anti-mouse IgG (Cappel Laboratory, USA) and lactoperoxidase (bovine milk, Sigma) were used.

Immunization and Fusions

Six weeks-old male mouse (Balb/c) received an intraperitoneal injection of crude horse-radish peroxidase (Type I, 1 mg) in 0.3 ml of complete Freund adjuvant. Booster injections (1 mg of the same enzyme) were given intraperitoneally at 2 and 3 weeks, respectively, after the first injection. After 1 week, the splenocyte was fused with

mouse myeloma line (P3-X63-U1 AZG^R), which was a generous gift from Prof. T. Watanabe (Saga Medical University), according to the method (Milstein, 1981). The serum from the sacrificed mouse was used for analysis in the further experiments (Tables 1 and 2). The spleen cells (2×10^7) were fused with mouse myeloma cells (2×10^6) by incubation for 1 min with 1 ml of 42.5% polyethyleneglycol 1000 and 15% of dimethylsulfoxide. Then, they were diluted stepwisely with 9 ml of MEM medium (Flow Lab.). After centrifugation (800 rpm for 10 min), the cells were rediluted with 10 ml of hypoxanthine/aminopterin/thymidine (HAT) selection medium, and divided into 50 wells (0.2 ml each). After four half replacements of the selective medium were done during the next two weeks, the supernatant of each well was assayed.

Ouchterlony Method

Agar (1.2%) in a Veronal buffer (pH 8.6) was coated on glass plates. 1 or 3 mm-diameter wells were punched, and enzyme or antiserum was applied to it.

Passive Hemagglutination Test

For the antibody against horseradish peroxidase, the following test was performed. Tannic acids (12 mg/100 ml saline) was incubated with sheep red blood cells (SRBC) (6% of hematocrit value) at 37°C for 20 min. After washing with saline, the SRBC was suspended to 6% of hematocrit value with 0.15 M citrate- Na_2HPO_4 (pH 5.0). Then, an equal volume of peroxidase (Type I, Sigma) (50 $\mu\text{g}/\text{ml}$ saline) was incubated with above-mentioned SRBC solution for 90 min at 39°C and stirred at room temperature overnight. After washing the enzyme-fixed SRBC with saline, the passive hemagglutination test was performed. 25 μl of stepwisely-diluted hybridoma supernatant and the same volume of enzyme-fixed SRBC was incubated at 37°C in a multititer plate (Falcon) and added to the mixture with sheep IgG fraction anti-mouse IgG (4 $\mu\text{g}/\text{well}$).

Assay for Peroxidase Activity

Peroxidase activity was assayed by a slight modification of Bergmeyer *et al.* (1974). 20 μl of 30% of H_2O_2 and original guaiacol (Nakarai Chemicals) was dissolved in to 50 ml of phosphate buffer saline (10 mM, pH 7.2), and the solution was further diluted to 10-fold with the same PBS and 2 μg of each enzyme and 1 ml of the above-mentioned substrate solution were mixed and stood for 2 min at room temperature. Enzyme activity was determined from the increase of absorbance at 436 nm. In the case for the assay of hybridoma supernatant, the sample was concentrated by salting out with $(\text{NH}_4)_2\text{SO}_4$ (60% saturation, pH 7.5).

Results and Discussions

Although the seven horseradish peroxidase isozymes have been already reported (Shannon

Fig. 1. Immunodiffusion analysis of various peroxidases. $2\text{ }\mu\text{g}$ of each enzyme and $5\text{ }\mu\text{g}$ of bovine serum albumin were applied to each well (1 mm diameter), respectively. The center well contained goat anti-peroxidase serum ($1\text{ }\mu\text{g}$ of antibody).

et al., 1966), the details of their antigenicities have not been elucidated. As a preliminary experiment, the reactivity of horseradish peroxidase isozyme against the goat anti-peroxidase serum was analyzed by using the ouchterlony method. As shown in Fig. 1, Type II and IX enzymes have a common precipitation line and Type VII and VIII isozymes represent weak spur lines from the former common line. In addition, goat and mouse anti-peroxidase serums repressed all the tested isozyme reactions (Type VII, VIII and IX) to the same extent (Table 2a). These results suggest that these conventional serums could not differentiate isozyme-specific antigen sites.

Next, in order to obtain a specific antibody against peroxidase isozyme, the hybridoma cells between enzyme-immunized splenocyte and myeloma, P3-X63-U1 AZG^R were prepared after the selection by the HAT (hypoxanthine, aminopterin and thymidine) medium. As a result, two clones (B7 and G6) having antibody activity against peroxidase were detected (Table 1). Furthermore, the secreted antibodies of these two clones repressed specifically Type VIII isozyme activity (Table 2b) and the dose responses for Type VIII isozyme were analyzed (Fig. 2). Also, the relationship between the inhibitory activity and the binding to enzyme was studied. After, the antibody was incubated with the enzyme, the enzyme-antibody complex was precipitated with the second antibody (rabbit anti-mouse IgG serum). Then, the free peroxidase activity in the supernatant was assayed. The result clearly demonstrated that there is a parallel relationship between the decrease of enzyme activity and the amount of added antibody (Fig. 3), which suggests that the binding of the specific antibody to Type VIII isozyme affects its activity.

Fig. 2. Inhibitory effect by hybridoma-producing antibody for Type VIII isozyme activity. Added antibody was prepared by concentration with 50% $(\text{NH}_4)_2\text{SO}_4$ saturation of each culture supernatant. The assay condition was the same as in Table 2b.

Fig. 3. Binding of hybridoma-producing antibody for Type VIII isozyme. The assay mixture contained 2 μg of Type VIII enzyme, which was added to 20 μl of B7 antibody or 30 μl of G6 antibody. After incubation for 1 hr at 37°C, the antigen and antibody complex was co-precipitated with rabbit anti-mouse IgG serum (almost saturable amount) and non-specific mouse IgG (2 μg). After centrifugation, the free enzyme activity in the supernatant fraction was assayed. Then, the same treatment was repeated twice more.

Previously, Zagury *et al.* (1979) reported the monoclonal antibody against horseradish peroxidase by the cell fusion technique, but this antibody was not analyzed concerning specific reactivity for isozymes. The prepared antibodies as noted in this paper can differentiate isozyme-specific antigen sites, which are responsible for enzyme activity. Thus, the specific antibody produced by hybridoma may be a useful tool for the study of horeseradish peroxidases and their applications.

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ハイブリドーマによる西洋ワサビパーオキシダーゼ・アイソザイム
に対する特異抗体の産生

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要 旨： 西洋ワサビパーオキシダーゼは、現在、細胞生物学などの分野において細胞追跡あるいは酵素抗体法などに使われる重要なマーカーエンザイムとして知られている。しかし現在用いられている諸方法をより選択的かつ正確な方法とするために著者らは、本酵素のアイソザイムに対して特異的に反応する抗体の作成を試みた。本酵素に対する市販の抗血清は、種々のアイソザイムを特異的に識別できなかったが、本酵素を免疫したマウスの脾細胞とミエロマ細胞をポリエチレングリコールにより融合させアイソザイムに特異的な抗体を産生する細胞株の作成に成功した。作成された抗体は、アイソザイム・タイプⅧにのみ選択的に反応しその酵素活性を抑制した。

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