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Immunoenzymometric assay for recombinant methioninase in biological fluids

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Abstract

Immunoassay for recombinant methioninase (rMETase), an anti-cancer agent, in biological sample was developed. Antisera were produced by immunizing rabbits with rMETase. The antisera were evaluated using radioiodine-labeled rMETase and good antisera for sensitive immunoassay were obtained. Horseradish peroxidase (HRP) was coupled to reduced IgG of K232 antiserum through bridging agent, *N*-(ε-maleimidocaproyloxy) sulfosuccinimide ester (sulfo-EMCS), to prepare enzyme-labeled antibody. IgG fraction of K231 antiserum was immobilized on microplate well. Two-site sandwich immunoenzymometric assay (IEMA) was developed using these antibodies and had good standard curve between 0.4 and 12 ng per well. For determination of rMETase in mouse plasma, sample was diluted 100-fold with dilution buffer containing protease inhibitors, because about 10% of rMETase immunoreactivity was lost for 2 h at room temperature. rMETase in mouse plasma could be determined by the proposed method in the range of 0.5–8 μg/ml and the method was validated. This novel IEMA, in substitution for the measurement of its enzyme activity, should be very useful not only for preclinical studies of rMETase but also for the clinical studies.

Keywords: Anti-cancer agent; Methioninase; Immunoenzymometric assay; Horseradish peroxidase; Mouse plasma

1. Introduction

Tumor-specific increase of minimal requirement for methionine was found [1–3] and a methionine cleaving enzyme, methioninase, is expected to be useful for cancer therapy [4,5]. Recently, recombinant methioninase (rMETase, MW 172 000) has been obtained by large-scale production using *Escherichia coli* [6] and is now under development for an anti-cancer agent in our laboratory.

It is necessary to measure rMETase concentration in biological fluids obtained from pre-clinical and clinical studies, however, there is only a method for the enzyme activity, which is not very sensitive for biological samples [6,7]. Immunoassay is the most suitable assay method for determination of proteins in biological matrices. Thus, we prepared anti-rMETase antisera using rabbits and evaluated the antisera by radioiodine-labeled rMETase. Antibodies purified from the

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antisera were coupled with horseradish peroxidase (HRP) and 2-site sandwich immunoenzymometric assay (IEMA) was developed. The IEMA was optimized for rMETase measurement in mouse plasma samples and the method was validated.

2. Experimental

2.1. Reagents

Purified rMETase was obtained from Shionogi & Co., Ltd. [5]. Other reagents were commercially available special grade materials, unless otherwise specified.

2.2. Preparation of antiserum

Immunogen, approximately 3 mg of rMETase in 0.1 ml, was diluted with 3 ml of saline and 1.5 ml of the solution was entirely mixed with 1.5 ml of Freund's complete adjuvant (FCA, Difco Lab., Detroit, MI, USA) to make emulsion. The rest of rMETase solution, 1.0 ml, was added to 1.0 ml of Ribi adjuvant system (RAS, Funakoshi, Tokyo, Japan) and mixed well. Half a milliliter of the emulsion or the mixture containing approximately 0.25 mg of rMETase was injected subcutaneously into 10–20 sites on the back of each of five Japanese white rabbits (Kitayama Labesu, Ina, Japan). Immunization was repeated every 3 weeks and small amount of blood was collected 10 days after the third and forth immunization. Whole blood was collected 10 days after fifth immunization (Serum No. K229-231 using FCA and K232-233 using RAS).

2.3. Radioiodine-labeled rMETase

Radioiodine-labeled antigen was prepared by chloramine T method. [8] Radioiodine solution (NEN, Wilmington, DE, USA), 0.002–0.0025 ml (9.25 MBq), and 0.010 ml of chloramine T solution (2 mg/ml) in phosphate buffer (pH7.5, 0.5 M) were sequentially added to a small polypropylene tube (Treff, Degersheim, Switzerland) which contained 0.050 ml of phosphate buffer (pH7.5, 0.5 M) and 0.020 ml (approximately 0.01 mg) of protein (rMETase or IgG). The reaction solution was mixed for 30 s and, then, 0.050 ml of sodium metabisulfite solution (250 mg/ml) in phosphate buffer (pH7.5, 0.5 M) was added to the mixture to stop the reaction. Further, 0.010 ml of potassium iodide aqueous solution (100 mg/ml) and 0.010 ml of bovine serum albumin (BSA, Sigma, St. Louis, MO, USA) aqueous solution (10 mg/ml) were added and mixed. The reactant was applied to a short column of Sephadex G-25 (PD-10, Amersham Pharmacia, Uppsala, Sweden) and radioactive fractions of high-molecular weight were collected (eluent: phosphate buffer (pH 7.4, 0.1 M)).

2.4. Evaluation of antiserum by competitive RIA

A phosphate buffer (pH7.4, 0.1 M) containing sodium chloride (0.05 M), BSA (0.5 g/dl) and sodium azide (0.05 g/dl) was used throughout this radioimmunoassay (RIA) for reagent dilution (RIA buffer). A 0.100 ml portion of the diluted iodine-labeled antigen solution and 0.100 ml of the diluted antiserum were added to a polystyrene tube (Shionogi tube, Shionogi, Osaka, Japan) containing 0.100 ml of diluted normal rabbit plasma (Dailchi RI Lab., Tokyo, Japan) or 0.100 ml of rMETase standard solution diluted with the normal rabbit plasma. After mixing, the mixture was stood at 4 °C for 16 h (first reaction). Next, 0.100 ml of anti-rabbit second antibody solution (Daiich RI Lab.) was added and further stood at 4 °C for 16 h (second reaction). The mixture was centrifuged at $2000 \times g$ for 30 min after the second reaction, the supernatant was aspirated off and the radioactivity of the residue was counted with a gamma counter (ARC-600, Aloka, Tokyo, Japan).

Antiserum was evaluated by antiserum dilution curve in which 0.100 ml of diluted radioiodine-labeled antigen was added to an assay tube containing 0.100 ml of several dilutions of antiserum and 0.100 ml of the diluted normal rabbit serum. The procedure was the same as described above except first reaction conditions at room temperature for 16 h and second reaction conditions at room temperature for 24 h.

2.5. Purification of antiserum

Antiserum was purified by the usual way [9,10]. Briefly, γ-globulin was precipitated by adding a portion of saturated ammonium sulfate solution to two portions of the antiserum (K229, K230, K231, K232 or K233) and standing in ice water for 20 min. After centrifugation, the precipitate was dissolved in distilled water and concentrated by centrifugal concentrator (Centricon YM-30, Millipore, Bedford, MA, USA).

The γ-globulin solution was applied to Protein A affinity column (Affi-Gel protein A MAPS II kit, Bio-Rad, Hercules, CA, USA) and IgG was eluted with the elution buffer of the kit after washing with the wash buffer of the kit. The IgG fraction was concentrated by the centrifugal concentrator.

2.6. Preparation of enzyme-labeled antibody

Mercaptoethylamine solution (0.1 M), 0.025 ml, was added to approximately 0.32 mg of K232 antibody (IgG) in 1 ml of phosphate buffer (pH6.0, 0.1 M) containing EDTA (5 mM) and reduced the antibody at room temperature for 1.5 h. The reactant was applied into PD-10 column (eluent: phosphate buffer (pH6.0, 0.1 M) containing EDTA (5 mM)) and an eluate of high molecular weight fraction was concentrated by the centrifugal concentrator to obtain reduced IgG.

N-(ε-Maleimidocaproyloxy) sulfosuccinimide ester (sulfo-EMCS, Pierce, Rockford, IL, USA) [11], 0.1 mg, in 0.05 ml of distilled water was added to 1.0 mg of HRP (approximately 50 nmol, EIA grade, Boehringer Mannheim, Mannheim, Germany) in 0.20 ml of phosphate buffer (pH 7.0, 0.1 M) and reacted at room temperature for 2 h to introduce maleimide residue at amino groups on HRP. The reactant was purified by PD-10 column (eluent: phosphate buffer (pH 6.0, 0.1 M) containing EDTA (5 mM)) and eluate of high molecular weight fraction was concentrated by the centrifugal concentrator to obtain maleimide activated HRP.

The maleimide activated HRP, 0.026 ml (0.128 mg), was mixed with 0.13 mg of the reduced IgG in 0.25 ml of phosphate buffer (pH 6.0, 0.1 M) containing EDTA (5 mM) and reacted at room temperature for 16 h. The reactant, 0.15 ml, was purified with gel-permeation HPLC (system: L-7100, Hitachi, Tokyo, Japan; column: TSKgel G3000SW$_{XL}$, Tosoh, Tokyo, Japan, 300 × 6.8 mm; eluent: phosphate buffer (pH 7.2, 0.1 M) containing sodium chloride (0.2 M); flow rate: 0.5 ml/min; detection: 403 nm). Fraction between 14.0 and 15.3 min of retention time was collected and used as enzyme-labeled antibody after dilution with an IEMA buffer described below.

2.7. Immunoenzymometric assay

The IgG solution (0.01 mg/ml) from K231 antiserum in phosphate buffer (pH 7.4, 0.1 M) containing sodium chloride (0.05 M) (IEMA coating buffer), 0.100 ml, was pipetted into a well of 96-well microplate (SUMIRON H Type, Sumitomo Bakelite, Tokyo, Japan) and the well was stood at room temperature for about 16 h. Then, the well was blocked with 0.2 ml of the IEMA coating buffer containing BSA (0.5 g/dl) (IEMA buffer) at room temperature for more than 2 h after twice washing with 0.2 ml of the IEMA buffer. The buffer was entirely aspirated off just before use as IgG immobilized well.

Mouse plasma sample was 100-fold diluted with the IEMA buffer containing aprotinin (500 KIU/ml) and EDTA 2Na (0.125 g/dl) (sample dilution buffer). The diluted sample or rMETase standard solution (0 and 0.4–12.5 ng per well, prepared with the sample dilution buffer containing 1% mouse plasma), 0.100 ml each, was pipetted in double into the IgG immobilized well. The well was stood at room temperature for more than 5 h (first incubation). The diluted enzyme-labeled antibody (50 ng/ml of K232 IgG labeled with HRP by sulfo-EMCS) solution, 0.100 ml, was added and the well was stood at room temperature for more than 16 h (second incubation) after washing three times with 0.2 ml of the IEMA buffer. Enzyme substrate solution (TMB+, DAKO, Carpinteria, CA, USA), 0.100 ml, was added to the well after washing three times with the IEMA buffer and mixed well. The well was incubated at room temperature for 30 min and the enzyme reaction was stopped with addition of sulfuric

Table 1
Titer of rabbit anti-rMETase antiserum

Immunization	Titer ($\times 10^3$)				
	K229	K230	K231	K232*	K233*
Third	300	300	350	300	350
Fourth	450	400	650	550	400
Fifth	220	180	270	300	200

K229, K230, K231: FCA K232*, K233*: RAS.

acid (0.5 M). The absorbance at 450 nm of the each well was measured with a multilabel counter ($ARVO_{SX}$ 1420, Wallac Oy., Turku, Finland).

3. Results and discussion

3.1. Preparation of antiserum and its evaluation by competitive RIA

Five antisera were obtained from 5 rabbits immunized with rMETase. rMETase was labeled with radioiodine (^{125}I) by chloramine T method successfully though iodination using chloramine T sometimes damages immunoreactivity of proteins by its strong oxidation potential. The labeled rMETase was used for evaluation of the antisera and changes of the labeled rMETase bound to the antibody were observed with varying dilution of the antisera. Table 1 shows dilutions of the antisera on a half of maximum bound percentages (40% to total count) (titer). The titer of every antibody after forth immunization was increased from that after third immunization but slightly decreased after fifth immunization (final immunization). The titer in final antiserum after fifth immunization was ranged from 180 000 to 300 000 with antigen. In addition, there were no apparent differences on production of antibody between two adjuvant systems for immunization.

From the standard curve of competitive RIA using each antiserum, 10% inhibition concentration (IC10%) and 50% inhibition concentration (IC50%) were estimated as shown in Table 2. The former concentration suggests approximate value of the limit of quantitation, 0.1–0.2 ng per tube (0.6–1.2 fmol per tube). Every antibody obtained here had high titer and good affinity and would be used for not only for detection or determination by immunochemical method but also for purification or isolation by immunoaffinity chromatography, immunoprecipitaion, et al. The results also suggest that the characteristics of the antisera were almost identical and immunogenesity of rMETase was strong.

3.2. Enzyme labeling and sandwich immunoenzymometric assay

Sandwich IEMA using microtiter plate is very popular for its easy operation without using radioisotopes [12]. K232 antibody was selected for the enzyme labeling and K231 antibody was used for immobilization because of its high titer though there was no significant difference in characteristics of five antisera described above.

K231 antiserum and K232 antiserum were purified to IgGs with protein A affinity column after precipitation by saturated ammonium sulfate solution. K232 IgG was further reduced by mercaptoethylamine and coupled with maleimide ester (sulfo-EMCS) derivative of the enzyme (HRP). The enzyme-labeled IgG was eluted earlier than untreated IgG on gel-permeation HPLC and the enzyme activity of the eluate was confirmed. The

Table 2
Parameters of RIA standard curve with various antisera

	K229	K230	K231	K232	K233
Dilution of antiserum	250 000	200 000	300 000	300 000	200 000
0 conc. Binding (Bo/T) (%)	33	31	33	36	38
IC 10% (ng per tube)	0.1	0.2	0.2	0.2	0.2
IC 50% (ng per tube)	1.5	2.5	2	3	2

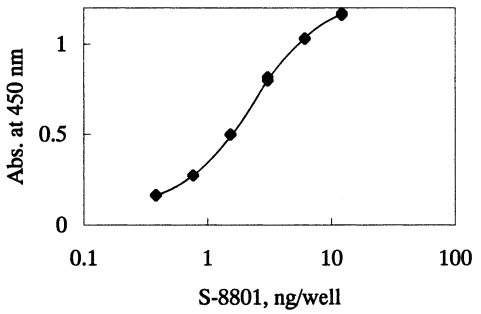

Fig. 1. IEMA standard curve of rMETase.

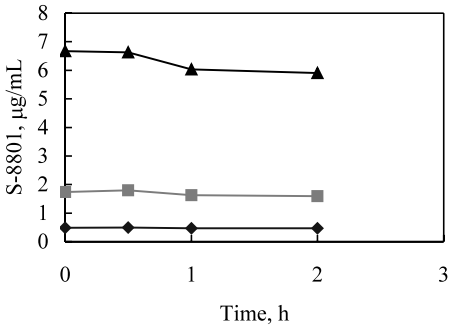

Fig. 2. Stability of rMETase in mouse plasma at room temperature. Control samples: high (▲), medium (■) and low (◆).

IEMA standard curve using the enzyme-labeled IgG of K232 and immobilized antibody of K231 had good response ranged from 0.4 to 12 ng per well (Fig. 1).

3.3. IEMA for rMETase in mouse plasma

Recently, mice were used for pharmacological studies of this drug [5] and IEMA for rMETase in mouse plasma was developed although species for drug safety study was not planned. rMETase was not very stable and its immunoreactivity gradually decreased to about 90% for 2 h (Fig. 2). Thus, the plasma sample was immediately diluted 100-fold with the sample dilution buffer containing protein inhibitors just after the blood collection. In addition, rMETase standard solution was constructed of the sample dilution buffer containing 1% mouse plasma. Standard curve for mouse plasma sample was ranged from 0.4 to 12 μg/ml after 100-fold dilution and should become more sensitive by smaller dilution, if necessary.

3.4. Precision and accuracy

Precision and accuracy of the proposed IEMA were estimated. As shown in Table 3, between-run precision and accuracy were small enough but not within-run (run 1). Almost within-run accuracy (bias) values in run 1 were slightly small, which was not found in run 2 and run 3, and, thus, it was thought to be temporal variation. The limit of quantitation was 0.5 μg/ml and the assay range was between 0.5 and 8 μg/ml.

3.5. Stability of rMETase in mouse plasma

rMETase was added to mouse plasma to prepare spiked sample. The spiked samples were 100-fold diluted immediately with the dilution buffer and the stability of rMETase immunoreactivity in the diluted samples was investigated. Tables 4 and 5 show short term stability at −40 and 4 °C and repeated freeze–thaw stability. Results about short term stability suggest that

Table 3
Precision and accuracy of IEMA

Run	Added (μg/ml)	Mean ± S.D. (μg/ml)	*n*	Bias (%)	R.S.D. (%)
Within-run (Run: 1)	0.51	0.488 ± 0.008	6	−4.3	1.6
	2.04	1.74 ± 0.03	6	−14.7	1.7
	8.16	6.67 ± 0.11	6	−18.3	1.6
Between-run (Run: 1–3)	0.51	0.531 ± 0.038	18	4.1	7.2
	2.04	1.96 ± 0.22	18	−3.9	11.2
	8.16	7.34 ± 0.82	18	−10.0	11.2

Table 4
Short-term stability

	Spiked plasma (μg/ml)	Remained (%)		
		Day 0	Day 1	Day 7
−40 °C ($n = 6$)	0.51 (100%)	95.7	112.4	104.3
	2.04 (100%)	85.3	105.9	96.6
	8.16 (100%)	81.7	102.2	85.8
4 °C ($n = 3$)	0.51 (100%)	95.7	116.9	123.1
	2.04 (100%)	85.3	108.3	108.3
	8.16 (100%)	81.7	103.3	92.8

Table 5
Freeze–thaw stability

	Spiked plasma (μg/ml)	Remained (%)		
		Cycle 0	Cycle 1	Cycle 3
Freeze–thaw	0.51 (100%)	95.7	110.0	108.4
	2.04 (100%)	85.3	106.4	94.6
	8.16 (100%)	81.7	104.0	78.1

rMETase in the dilution buffer was stable for 1 week not only at −40 °C but also at 4 °C although some variations were found in the measured values which did not have any tendencies. The spiked samples after dilution with the dilution buffer were also stable under three freeze–thaw cycles.

4. Conclusions

The IEMA using the immobilized antibody on microplate and the HRP labeled antibody through the sulfo-EMCS bridge had good standard curve ranged between 0.4 and 12 ng per well. rMETase in mouse plasma was not very stable and had to immediately dilute 100-fold with the dilution buffer containing EDTA and aprotinin. The IEMA could determine rMETase, from 0.5 to 8 μg/ml, in mouse plasma easily.

Our novel IEMA for rMETase, in substitution for the measurement of its enzyme activity, should be very useful not only for the preclinical studies but also for the clinical studies.

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