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Enzyme Immunoassay for N^{α}-(*N*-Acetylmuramylalanyl-D-isoglutaminyl)-N^{ε}-stearyllysine

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An enzyme immunoassay method for N^{α}-(*N*-acetylmuramylalanyl-D-isoglutaminyl)-N^{ε}-stearyllysine (MDP-Lys-L18) was established.

A heterologous system was adopted based on the enzyme-labeled conjugate (EC) prepared by linking MDP-lysine (MDPL; an analogue of MDP-Lys-L18) to horseradish peroxidase (HRP) (EC 1.11.1.7) by the sodium metaperiodate method, and anti-MDP-Lys-L18 antiserum obtained from a rabbit that had been immunized with MDP-Lys-L18 conjugated with bovine serum albumin (BSA) by the carbodiimide method. The bound and free ECs were separated by a double antibody method. The lower limit for the quantitative determination of MDP-Lys-L18 in serum was estimated to be 100 pg/ml and the coefficients of variation of this assay (intra-assay) were between 3 and 8%. This enzyme immunoassay is superior in sensitivity to the radioimmunoassay previously reported. Serum concentrations of MDP-Lys-L18, after subcutaneous administration at a dose of 50 μg/kg to rats, were determined by means of this assay.

Keywords——enzyme immunoassay; muramyl dipeptide; enzyme-labeled conjugate; sodium metaperiodate; horseradish peroxidase; heterologous system; double antibody method

N^{α}-(*N*-Acetylmuramylalanyl-D-isoglutaminyl)-N^{ε}-stearyllysine (MDP-Lys-L18, Fig. 1) is a derivative of muramyl dipeptide (MDP; *N*-acetylmuramyl-L-alanyl-D-isoglutamine), which has been reported to have an immunomodulating effect in experimental animals.[1] In order to develop a method for quantitative analysis of MDP-Lys-L18 in biological fluids, we previously investigated a radioimmunoassay (RIA),[2] in which MDP-Lys-L18 was found to have the property of binding non-specifically to protein co-existing in the system owing to the stearyl residue in the molecule; this binding decreased the sensitivity of the RIA. Additionally, in RIA, the sensitivity is generally greatly restricted by th specific radioactivity of the labeled compound used for competition. These considerations suggested that there was still room for improving the sensitivity of the immunoassay for MDP-Lys-L18. The present paper deals with an enzyme immunoassay (EIA) for MDP-Lys-L18 based on a "hapten" and "site"

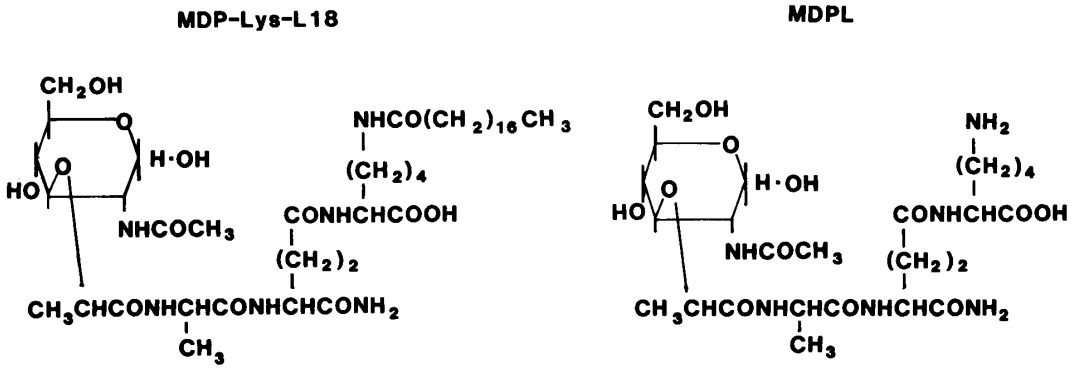

Fig. 1. Structure of MDP-Lys-L18 and MDPL

heterologous system, in which MDP-lysine (MDPL, Fig. 1), an analogue compound of MDP-Lys-L18, was coupled to horseradish peroxidase (HRP) (EC 1.11.1.7) and used as enzyme-labeled conjugate (EC) in order to decrease the affinity for binding of EC to antibody and to reduce the unfavorable effect of the stearyl residue, with the aim of obtaining a highly sensitive assay method.

Materials and Methods

Reagents——MDP-Lys-L18, MDP, MDPL and *N*-acetylmuramic acid (NAM) were provided by the Laboratory of Medicinal Chemistry, Research Institute, Daiichi Seiyaku Co., Ltd. HRP was purchased from Calbiochem-Behring, and 3-(*p*-hydroxyphenyl)propionic acid (HPPA) and human serum albumin (HSA) from Sigma Chemical Co. Sodium metaperiodate and sodium borohydride were obtained from Nakarai Chemicals Ltd. Goat anti-rabbit IgG was purchased from Medical and Biological Laboratories Ltd. Sephacryl S-200 was provided by Pharmacia Fine Chemicals. The other reagents used were of analytical grade. Anti-MDP-Lys-L18 antiserum was prepared in the rabbit by immunization with MDP-Lys-L18 conjugated with BSA in the manner reported in the previous paper.[2] Disposable test tubes were from Toyo Kizai Kagaku Ltd.

Preparation of Enzyme Conjugate (HRP–MDPL Conjugate)——MDPL was conjugated with HRP (4 mg) by using sodium metaperiodate according to the method of Wilson and Nakane.[3] Four different ECs (EC I—IV) were prepared at the coupling molar ratios (MDPL/HRP) of 5 (EC I), 15 (EC II), 30 (EC III) and 60 (EC IV). After the conjugation reaction, each resulting solution was dialyzed against phosphate-buffered saline (PBS, pH 7.3) at 4 °C and further fractionated on a Sephacryl S-200 column (2.6 × 90 cm). The absorbance at 403 nm of each fraction was measured. In order to estimate the molecular weight of EC, HSA (M_r 70000) was chromatographed on the same column separately and its elution pattern was analyzed by measuring the optical density (OD) at 280 nm. The fraction corresponding to the desired conjugate peak was pooled and stored according to the method of Wilson and Nakane.[3] The content of MDPL in each EC was determined by amino acid analysis as reported in the previous paper.[2] The number of MDPL residues per molecule of HRP was calculated by means of the following equation; [molar ratio of Ala/Leu in EC − molar ratio of Ala/Leu in HRP] × 35.[4]

On the other hand, the EC fraction from the Sephacryl S-200 column was assayed for binding activity to anti-MDP-Lys-L18 as follows: ten microliters of each fraction was incubated with 400 μl of diluted (1/1600) antiserum to MDP-Lys-L18, and the enzyme activity of the conjugate bound to anti-MDP-Lys-L18 was measured according to the procedure described below under "Enzyme Immunoassay."

Association Constant for Binding of Antibody with EC——In order to estimate the association constant (K_a) for binding of anti-MDP-Lys-L18 with EC, 500 μl of diluted (1/2000) antiserum was incubated with EC (1.0×10^{-10}—2.0×10^{-8} M) for 72 h at 4 °C. The molar concentration of EC used was calculated from the absorbance at 403 nm and *E*-value of HRP at 403 nm ($E^{1\%}_{1\,cm} = 22.75$).

The separation of the bound EC from the free EC was accomplished by a double antibody method, and the enzyme activity of the bound EC was measured as described under "Enzyme Immunoassay."

From the enzyme activity of the immune precipitate, the molar concentration of the bound EC was calculated using the standard curve for HRP, which was prepared with known amounts of standard HRP by measuring the enzyme activity under the same conditions as used for the samples. The K_a for binding of anti-MDP-Lys-L18 with EC was determined according to the procedure reported by Scatchard.[5]

Enzyme Immunoassay——Ten-milliliter plastic disposable tubes were used in the assay. Dilution of antiserum or EC was done with PBS containing 0.002% Timerosal. Each tube contained 50 or 100 μl of blank serum or test sample, 100 μl of diluted EC in PBS containing 0.1% HSA, 100 μl of diluted antiserum, and 200 μl of MDP-Lys-L18, its analogue or none in PBS containing 0.1% HSA. The mixture was incubated for 72 h at 4 °C. In order to separate HRP–MDPL conjugate bound to antibody from the free conjugate, 150 μl of diluted (1/20) goat anti-rabbit IgG antiserum in PBS and 50 μl of 1% normal rabbit serum in PBS containing 0.05 M ethylenediaminetetraacetic acid (EDTA) were added to the mixture, which was further incubated for 48 h at 4 °C, and then centrifuged at 3000 rpm for 15 min at 4 °C. The supernatant was removed by aspiration, and the precipitate was washed once with chilled water.

The precipitate was suspended in 200 μl of PBS, and the enzyme activity of HRP was measured by the fluorimetric method of Ishikawa *et al.*[6] using HPPA and hydrogen peroxide as substrates.

Incubation was conducted for 15 min at room temperature. MDP, MDPL and NAM were employed to investigate the inhibition of the assay by compounds related to MDP-Lys-L18.

Animal Experiments——Male Wistar-Imamichi rats (body wt. 280—310 g, purchased from the Institute of Animal Reproduction) were injected subcutaneously on the dorsum with MDP-Lys-L18 dissolved in saline at a dose of 50 μg/kg/ml. The serum samples were collected at 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, and 24.0 h after administration. The number of rats in each group was 3. The serum was separated by centrifugation and stored at −20 °C until

analyzed. For assay, 50 μl aliquots of the serum were worked up by the procedure described under "Enzyme Immunoassay."

Results

Enzyme-Labeled Conjugates

The Sephacryl S-200 elution pattern of a typical HRP-MDPL conjugation mixture is shown in Fig. 2. HRP content is indicated in terms of the optical density at 403 nm. Every elution pattern showed two typical peaks, fraction A and fraction B, irrespective of the coupling ratio. Fraction A had a higher molecular weight (M_r) than HSA (M_r 70000), while fraction B had a lower M_r. From its molecular weight, the compound in fraction A was considered to be the self-coupled enzyme, because the M_r of HRP is reported to be 40000.[7] Table I shows the results of amino acid analysis of conjugation mixtures, fraction A and fraction B, of EC I—IV. The number of MDPL molecules conjugated with HRP (MDPL/HRP ratio) increases in proportion to the increase of coupling ratio. However there was a tendency for the yield of the conjugation reaction to become lower as the coupling ratio increased. The molar ratio of Ala/Leu (0.66) determined after hydrolysis of HRP was nearly equal to the value obtained by calculation based on the reported numbers of Ala (23) and Leu (35) residues in the HRP molecule.[4] Table II shows the relation between the MDPL/HRP ratio and the immunoreactivity with anti-MDP-Lys-L18 for both fraction A and fraction B of each EC. It appears that the binding affinity of EC for antiserum increases as the MDPL/HRP ratio increases. Additionally, when fraction A and fraction B, from the same EC were compared, the former showed a higher affinity for the antibody than the latter, even though the MDPL/HRP ratio was nearly equal in both fractions.

Inhibition of Enzyme Activity by MDP-Lys-L18 in EIA

In the previous paper,[2] we reported that K_a for binding of anti-MDP-Lys-L18 antiserum with MDP-Lys-L18 was of the order of 10^8 to 10^9 M^{-1} (K_2). In this study, the apparent K_a for binding of anti-MDP-Lys-L18 antiserum with EC was found to be of the order of 10^6 to 10^8 M^{-1} (K_1). Accordingly, from these two association constants (K_1 and K_2), the inhibitory effect of MDP-Lys-L18 (I) on a competitive binding assay (EIA) was estimated in accordance

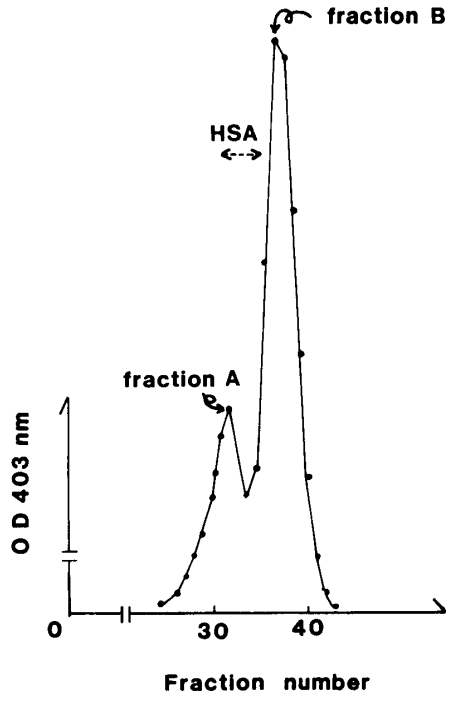

Fig. 2. Elution Pattern of a Typical HRP–MDPL Conjugation Mixture from a Sephacryl S-200 Column (2.6 × 90 cm) Eluted with PBS (1 Fraction, 7.5 ml)

HRP content is indicated in terms of the optical density at 403 nm.

TABLE I. Amino Acid Analysis of HRP and HRP–MDPL Conjugate

Hydrolyte (Coupling ratio)	Conjugation mixture Ala/Leu		MDPL/HRP (observed) Fraction A Ala/Leu		Fraction B Ala/Leu	
HRP	0.66		—		—	
Conjugate I (5)	0.72	$(0.72-0.66)\times 35^{a)}$ 2.10	0.71	$(0.71-0.66)\times 35^{a)}$ 1.75	0.73	$(0.73-0.66)\times 35^{a)}$ 2.45
Conjugate II (15)	0.75	$(0.75-0.66)\times 35^{a)}$ 3.15	0.80	$(0.80-0.66)\times 35^{a)}$ 4.90	0.79	$(0.79-0.66)\times 35^{a)}$ 4.55
Conjugate III (30)	0.78	$(0.78-0.66)\times 35^{a)}$ 4.20	0.91	$(0.91-0.66)\times 35^{a)}$ 8.75	0.82	$(0.82-0.66)\times 35^{a)}$ 5.60
Conjugate IV (60)	0.83	$(0.83-0.66)\times 35^{a)}$ 5.95	0.86	$(0.86-0.66)\times 35^{a)}$ 7.00	0.92	$(0.92-0.66)\times 35^{a)}$ 9.10

a) Reported number of Leu residues in HRP.

TABLE II. Characterization of Enzyme Conjugate in the Fraction

Conjugate (Coupling ratio)	MDPL/HRP[a)]	Bound[b)]/OD 403 nm
	Fraction A	
I (5)	1.75	13630
II (15)	4.90	16292
III (30)	8.75	20889
IV (60)	7.00	37426
	Fraction B	
I (5)	2.45	3607
II (15)	4.55	5318
III (30)	5.60	9597
IV (60)	9.10	14305

a) Calculated from the results of amino acid analysis. *b*) Fluorescence intensity due to enzyme activity of enzyme conjugate bound to diluted anti-MDP-Lys-L18 (1 : 1600).

with the following formula,

$$\text{inhibition}\ (\%)=\frac{[\mathrm{I}]}{[\mathrm{I}]+\frac{1}{K_2}(1+K_1[X])}$$

where $[X]$ is the amount of EC added. The appropriate amount of EC $[X]$ in the assay was calculated, and the proper amount of antiserum to give one-half of the maximum activity was also determined.

Though small differences in inhibition rate were recognized between calculation and observation, the inhibition curve prepared was very close to that anticipated from the calculation.

Enzyme Immunoassay

Table III shows the effect of the MDPL/HRP ratio in each EC on the quantitative characteristics of the EIA system. The binding ability of a definite amount of HRP

TABLE III. The Effect of the Ratio (MDPL/HRP) of Each Enzyme Conjugate on the Characteristics of EIA System

Conjugate (Fraction B)	MDPL/HRP[a]	Intensity[b]	50% inhibition value (MDP-Lys-L18, ng/tube)	MDPL cross reactivity (%)
I	2.45	1.0	<1	<10
II	4.55	2.3	1< <5	10< <20
III	5.60	6.4	5< <10	20< <30
IV	9.10	12.9	30<	100<

a) Calculated from the results of amino acid analysis. *b*) Relative fluorescence intensity due to enzyme activity of enzyme conjugate bound to anti-MDP-Lys-L18. EIA system: enzyme conjugate (absorbance at 403 nm: 0.001), anti-MDP-Lys-L18 (diluted 1 : 2500).

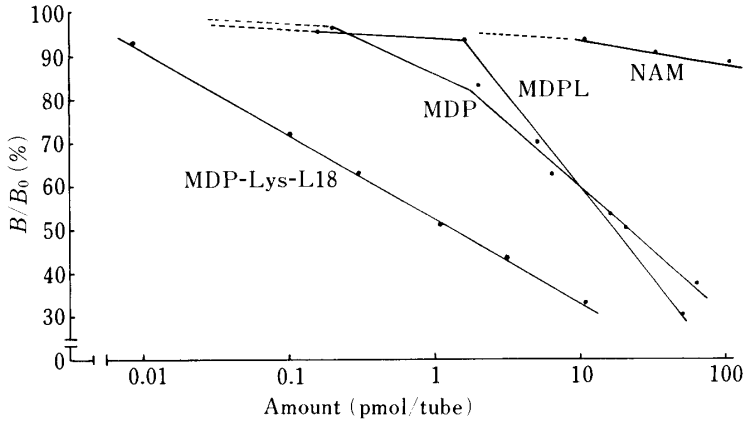

Fig. 3. Cross Reactivity of NAM, MDP and MDPL in the MDP-Lys-L18 EIA System Using the Diluted Enzyme Conjugate I-Fraction B (Absorbance at 403 nm; 0.001) and 1 : 2500 Diluted Antiserum

B and B_0 represent the fluorescence intensity bound in the presence and in the absence of MDP-Lys-L18 (or its derivatives), respectively.

(absorbance at 403 nm; 0.001) of each EC I—IV was determined at 1 : 2500 dilution of anti-MDP-Lys-L18 antiserum. The fluorecence intensity due to the enzyme activity of EC bound to anti-MDP-Lys-L18 increased as the MDPL/HRP ratio increased, but the amount of MDP-Lys-L18 required for 50% inhibition of the enzyme activity (50% inhibition value) also increased. This phenomenon seemed to be related to the degree of affinity of the EC for antibody. On the other hand, it was also recognized that the rate of cross reactivity of MDPL increased with increase in the MDPL/HRP ratio. It was concluded that EC I-fraction B would be the most favorable EC for the enzyme immunoassay. Figure 3 shows the standard curve for MDP-Lys-L18 and the inhibitory effects of MDP, MDPL and NAM on the assay when EC I-fraction B was used. When MDP-Lys-L18 was employed in the range from 10 fmol to 10 pmol per tube, the ratio B/B_0 decreased linearly as the concentration of MDP-Lys-L18 increased, and the slope of the regression curve was steeper than that observed in RIA. The blank value of each sample, which was large in the case of RIA, was quite small. Hardly any serum interference was observed. The lower limit of the quantitative determination for MDP-Lys-L18 in serum was estimated to be 100 pg/ml, so that the procedure is about five times more sentitive than RIA. The intra-assay variation coefficients were between 3 and 8%. Analogues, MDP and MDPL, showed about 10% cross reactivity with MDP-Lys-L18. NAM was less inhibitory. With regard to the cross reactivity of compounds related to MDP-Lys-L18, MDP, MDPL and NAM showed degrees of inhibition similar to those in RIA.

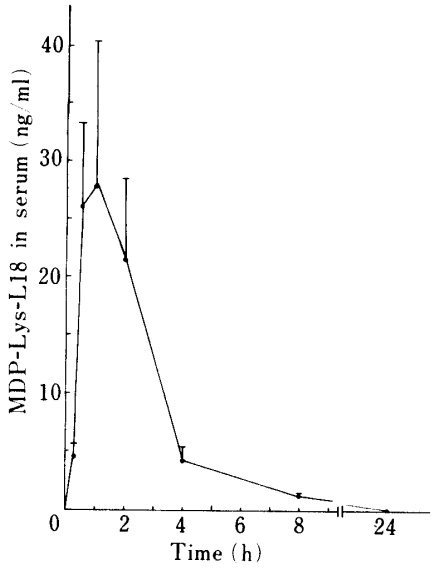

Fig. 4. Time Course of Serum Levels of MDP-Lys-L18 in Rats ($n=3$) after Subcutaneous Injection at a Dose of 50 μg/kg

Animal Experiments

The method developed in the present study was applied to the practical quantitative analysis of MDP-Lys-L18 in serum of rats.

The time course of the serum concentration of MDP-Lys-L18 after subcutaneous administration of the drug is shown in Fig. 4. The maximum serum concentration (C_{max}) was observed to be 27.3 ± 13.3 ng/ml serum (average $\pm$ S.E.) at 1 h after administration, though some variation was found among the three rats, and $t_{1/2}$ (biological half-life) and AUC (area under the concentration *versus* time curve) were calculated to be 0.97 h and 75.8 ng·h·ml^{-1}, respectively. No drug could be detected in the serum 24 h after administration.

Discussion

In order to develop an enzyme immunoassay method for MDP-Lys-L18, we attempted to prepare the EC by reacting a carboxyl group of MDP-Lys-L18 with the ε-amino groups of lysine residues of an enzyme (HRP or β-galactosidase), using carbodiimide or activated ester as a coupling agent. However these attempts were not successful, because, when the carbodiimide was employed, self-coupling of the enzyme appeared to take place to form an enzyme polymer, which bound non-specifically to protein and to the wall of the tube, so that the sensitivity of the competitive binding assay was lowered. On the other hand, when the *N*-hydroxysuccinimide ester of MDP-Lys-L18 was employed for coupling, the yield of the desired conjugate was extremely low, probably because the activated ester was unstable. Moreover, it was found that ECs thus prepared had such a high affinity for anti-MDP-Lys-L18 antiserum that MDP-Lys-L18 could hardly compete with them in binding to the antibody, as is often seen in homologous assays.

We therefore investigated the "hapten" and "site" heterologous system, where MDPL was conjugated with HRP by the method of Wilson and Nakane[3] and the conjugate was used as EC. Though Bahr *et al.* had already reported[8] the direct enzyme-linked immunosorbent assay (ELISA) using HRP linked with MDPL to detect the existence of anti-MDP antiserum and to examine its specificity, the reported assay method did not seem to be suitable for the quantitative analysis of MDP and nothing was stated about its sensitivity. In the present study, a highly sensitive binding assay method was achieved by the use of HRP–MDPL conjugate as EC. One of the factors that made the assay highly sensitive appeared to be the decrease in affinity of the EC for the antibody, caused by substitution of MDPL for MDP-

Lys-L18.

In the choice of enzyme to prepare EC, β-galactosidase was unsuitable because it was found to be unstable to freezing, which was indispensable for maintaining the stability of the MDPL residues coupled to the enzyme.

In this study it was observed that, as the number of MDPL molecules linked to the enzyme increased, the binding affinity for the antibody and the rate of cross reaction with MDPL were increased, and comparison of fraction A with fraction B showed the EC with higher M_r to have the higher affinity for the antiserum.

The reasons for these interesting phenomena are not clear.

As we reported in the previous paper,[2] the non-specific protein binding of MDP-Lys-L18, which was due to the hydrophobic stearyl residue in the molecule, influenced the sensitivity of the RIA. In the present study, the unfavorable effect of the stearyl residue on the sensitivity could be decreased to a considerable degree by employing a "site" heterologous EIA system.

As a result of examination of the inhibitory effect of MDP analogues on the assay, MDP, MDPL and NAM were found to be inhibitors, just as was noticed in RIA. However, as mentioned in the previous paper,[2] it seems unlikely that MDP and MDPL interfere with the assay for MDP-Lys-L18 in biological samples, because neither of them was found as a metabolite of MDP-Lys-L18 in mice and rats (unpublished data). Only NAM was detected as a metabolite (unpublished data), and its inhibitory effect was found by RIA to be so weak, and the amount formed as a metabolite was so minute, that its influence on the assay must have been negligible.

The enzyme immunoassay method developed in the present study was found to be applicable to the practical quantitative analysis of MDP-Lys-L18 in serum of rats. This method will be applied in the preclinical and clinical investigations on the pharmacokinetics and metabolic disposition of MDP-Lys-L18.

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