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MALEIMIDE DERIVATIVE OF HAPTEN FOR COUPLING TO ENZYME: A NEW METHOD IN ENZYME IMMUNOASSAY

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Summary

Meta-maleimidobenzoyl derivative of L-thyroxine methyl ester (MBTM) was synthesized and coupled to β-galactosidase at molar ratio of over 5 to 1. More than 97% of the enzyme was found to be labeled with MBTM as examined by double antibody precipitation method in excess of anti-T4 antibody. Maleimide group of MBTM was found to be labile; about 50% was destroyed in 3 hours when prepared in a solution of 1 μg/ml phosphate buffer (pH 7.0, 0.05M). With antiserum dilution of 2,400 fold, reproducible T4 enzyme immunoassay was carried out using double antibody precipitation method. A high sensitivity in the assay was observed on the 0-10 μg/100 ml range.

Introduction

We have synthesized a maleimidobenzoyl bridge for coupling small antigens to β-galactosidase with high yields and minimum loss of both enzyme activity and immunoreactivity. Maleimide derivatives have been used for coupling enzymes to proteins, such as immunoglobulins with N,N-o-phenylenedimaleimide (1,2) and insulin with m-maleimidobenzoyl-N-hydroxysuccinamide ester (3). Use of these coupling agents showed no appreciable reduction of enzyme activity due to the involvement of sulfhydryl groups of the enzyme in the coupling process.

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Coupling of haptens to the enzyme, on the other hand, is often identical to the preparation of hapten-protein conjugates for immunization. Such coupling involves either amino or carboxyl groups of the enzyme, resulting in either low efficiency of coupling (4) or reduction of enzyme activity (5). In order to avoid such disadvantages, we synthesized m-maleimidobenzoyl derivative of hapten for coupling to sulfhydryl groups of the enzyme. A high efficiency of binding to the enzyme without appreciable reduction in enzyme activity was found.

The present report describes the preparation of m-maleimidobenzoyl derivative of thyroxine (T4) methyl ester for the development of T4 enzyme immunoassay. We chose β-galactosidase because 1) it can be obtained in highly purified form, 2) it has a high catalytic number, 3) conjugation to sulfhydryl groups of the enzyme does not result in reduction of enzyme activity, 4) the enzyme and its conjugates can be stable up to one year when stored at 4°C, and 5) it is not present in human or animal biological fluids.

Materials and Methods

Chemicals. L-thyroxine and o-nitrophenyl-β-D-galactoside were obtained from Sigma Chemical Co., St. Louis, Missouri. Beta-galactosidase from E. coli was obtained from Boehringer Mannheim Biochemicals, Indianapolis, Indiana. Meta-aminobenzoic acid, benzaldehyde and maleic anhydride were obtained from Aldrich Chemical Co., Milwaukee, Wisconsin.

Antiserum. Antiserum to T4 was produced in rabbits by injection of T4 conjugated to bovine serum albumin prepared by the method of Gharib et al. (6). Final dilution of 1/400 was used in the assay. Cross reactivity with triiodothyroxine was minimal. Goat anti-rabbit immunoglobulin antibody was obtained from Calbiochem, La Jolla, California.

Synthesis of m-maleimidobenzoic acid (MBA). Meta-carboxymaleanilic acid was first prepared from meta-aminobenzoic acid and maleic anhydride following the method of Parola (7). It was then cyclized with acetic anhydride to give MBA following the procedure of Searle (8).

Synthesis of L-thyroxine methyl ester. L-thyroxine methyl ester was prepared by the method of Ashley and Harington (9).

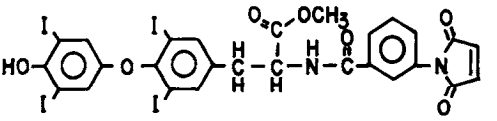

Figure 1. Structure of m-maleimidobenzoyl L-thyroxine methyl ester (MBTM).

Synthesis of m-maleimidobenzoyl derivative of L-thyroxine methyl ester. MBA (200 mg) was dissolved in 3 ml of thionyl chloride ($SOCl_2$) and refluxed for 30 minutes. Excess $SOCl_2$ was then evaporated under diminished pressure. The pale yellow powder of m-maleimidobenzoyl chloride (MBC) was kept overnight in a vacuum desiccator. The dried MBC was then dissolved in 10 ml of tetrahydrofuran (THF) and added dropwise to a stirred THF solution containing L-thyroxine methyl ester (400 mg) and a slurry of sodium carbonate (400 mg). The reaction mixture was refluxed for 30 minutes. At this point the linkage was complete and m-maleimidobenzoyl L-thyroxine methyl ester (MBTM, Fig. 1) produced was examined by thin layer chromatography (TLC) using Eastman chromatogram 13179 (Eastman Kodak Co., Rhochester, N.Y.) as an eluting plate and ethyl acetate as eluting solvent. The reaction mixture was then filtered and the solvent was removed under diminished pressure to yield crude pale-yellow product. The MBTM was purified by silica gel column chromatography (1.5 cm x 30 cm) using chloroform as eluting solvent. The isolated white powder of MBTM gave a single spot on TLC, Rf = 0.56, using ethyl acetate as solvent. The presence of maleimide group in the isolated product was confirmed by IR (Fig. 2) and by its ability to react with cysteine using the method of Grassetti and Murray (10). Melting points 137-141°.

Conjugation of β-galactosidase to maleimidobenzoyl L-thyroxine methyl ester (MBTM). Fifty microliters of a solution of MBTM in THF (0.2 mg/ml, 10 nmoles) was added to 1.5 ml of 0.05M phosphate buffer (pH 7.0) containing β-galactosidase (0.5 mg, 0.93 nmole). The mixture was incubated for two hours at room temperature. Following overnight dialysis in the same phosphate buffer, the mixture was chromatographed on a Sephadex G-25 column (1.5 x 40 cm). The fractions of eluate containing the peak of enzyme activity were used for the T4 assay. β-galactosidase activity was assayed by the method of Dray et al. (4) using o-nitrophenyl β-D-galactoside as substrate.

Thyroxine assay. Immunoassay for T4 was developed using MBTM-enzyme conjugate as label and second antibody for the separation of bound from unbound. To a glass tube (16 x 100 mm) 100 µl of a 100-fold dilution of the peak fraction of MBTM-enzyme conjugate eluted from Sephadex G-25 column was added, followed by addition of 500 µl of the phosphate buffer (pH 7.0, 0.05M) containing 0.5mM 8-anilino-1-naphthalene-sulfonic acid-sodium salt. Fifty µl of each of a series of standards containing 0, 2, 4, 8, 12 and 20 µg/100 ml of L-thyroxine in serum (Syva Corp., Palo Alto, Calif. 94303) was then added, followed by 100 µl of a 400-fold dilution of rabbit anti-thyroxine antiserum. The solution was mixed on a vortex mixer after each addition. After incubation

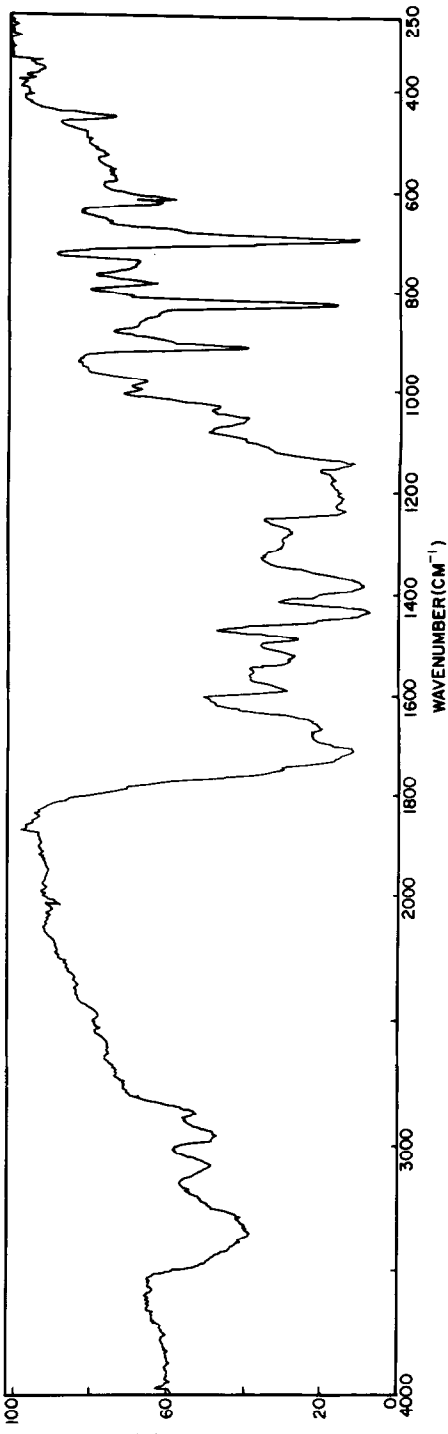

Figure 2. Infrared spectrum of MBTM (in KBr).

for 45 minutes at room temperature, 100 µl of 20-fold dilution of normal rabbit serum was added, followed by addition of 100 µl of goat anti-rabbit IgG. After vortex mixing, the tube was incubated for 15 minutes in ice water and centrifuged for 10 minutes at 3,000 rpm. The pellet was washed two times with the phosphate buffer and resuspended in 0.5 ml phosphate buffer-BSA (pH 7.5, 0.05M, 0.1% BSA). The enzyme activity was assayed using o-nitrophenyl β-D-galactoside as a substrate. Incubation time for enzyme activity was 45 minutes. Amount of o-nitrophenol produced at the end of incubation time was measured by Gilford Stasar III spectrophotometer at 420 mµ wavelength.

Results and Discussion

When MBTM and β-galactosidase conjugation was carried out at molar ratios of over 5 to 1, more than 97% of the enzyme was found to be conjugated with MBTM as examined by double antibody precipitation method in excess of anti-T4 antibody. The number of moles of MBTM conjugated per enzyme was not determined. Since it is known that β-galactosidase possesses about ten sulfhydryl groups per molecule (11), the maximum number of MBTM attached per enzyme is ten. The practical limit of solubility of MBTM in the conjugation solvent is 10 µg/ml, restricting the molar ratio of MBTM to enzyme. The maleimide group of MBTM was found to be labile; about 50% was destroyed in 3 hours when 1 µg of MBTM was dissolved in 1 ml phosphate buffer (pH 7.0, 0.05M). The conjugation reaction was, therefore, carried out immediately after dissolution of MBTM in the buffer. Enzyme activities examined before and after the conjugation step did not show any difference, suggesting full retention of the enzyme functional groups. With the final antiserum dilution of 2,400-fold, a reproducible T4 enzyme immunoassay was successfully demonstrated. Incubation times used in our assay were 45 and 15 minutes respectively for competitive binding of T4 and MBTM-enzyme conjugate and double antibody precipitation steps. The standard curve is shown in Fig. 3. The highest sensitivity in the assay was observed at 0-10 µg/100 ml range.

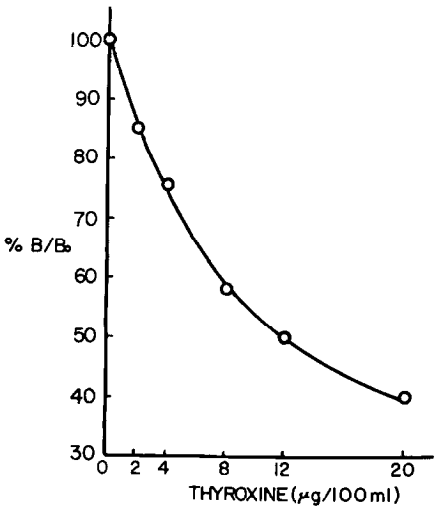

Figure 3. Standard curve for thyroxine enzyme immunoassay. Assay procedure is described in materials and methods section.

We believe that our novel approach in use of the maleimide derivative of hapten is a convenient and efficient way for conjugating hapten to an enzyme without reduction of enzyme activity. Our model of enzyme-hapten conjugation procedure could be extended to many haptens and can improve assay sensitivity and precision.

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