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Reversible fluorescence labeling of amino groups of protein using dansylaminomethylmaleic anhydride

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Abstract

The reversible fluorescence labeling of insulin, catalase and lysozyme has been demonstrated. As a derivatizing reagent, dansylaminomethylmaleic acid (DAM) has been used after investigating the precolumn and precapillary derivatization conditions. This reagent (DAM) reacts with the amino groups of proteins via its anhydride in the presence of a suitable dehydrating reagent, which then could be liberated under mild acidic conditions and the native proteins are regenerated. After the derivatization of insulin, catalase and lysozyme with DAM, no peaks of these native proteins were observed while several peaks of the derivatized proteins due to the multiple labeling were observed. However, after the regeneration, increasing amounts of the native proteins were observed as the regeneration period increased. For the lysozyme, the bacteriolytic activity of the enzyme decreased after the derivatization, and only 0.9% of the activity remained. The activity increases by the regeneration, and 95.6% of the bacteriolytic activity of the native enzyme was observed after a 48-h regeneration at pH 2.5 and 40 °C.

Keywords: Amino groups; Proteins; Dansylaminomethylmaleic anhydride

1. Introduction

Fluorescence labeling is a powerful tool in separation sciences for the sensitive determination of proteins and peptides. Until now, a number of methods have been developed using various derivatization reagents in combination with HPLC or HPCE [1]. A large number of these reagents react with amino groups to form stable derivatives. However, for the isolation and identification of new biologically active peptides and proteins, it should be better to recover the target molecules in their native, biologically active forms. As a result of the derivatization, proteins often change their biological activities [2,3], and none of the fluorescence labeling reagents have been reported to be dissociated from the derivatized analyte under conditions mild enough so that proteins are not denatured. Therefore, a reversible fluorescence derivatizing reagent, which enables both the sensitive detection of proteins and recovery in their native forms, is needed. In view of these facts, we have synthesized dansylaminomethylmaleic acid (DAM), a new fluorescence derivatization reagent for amines [4]. This reagent has a dansyl (1-dimethylaminonaphthalene-5-sulfonyl) group as a fluorophore, and also has a methylmaleic acid moiety, which is easily dehydrated in the presence of a suitable dehydrating reagent. The

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methylmaleic anhydride moiety is suitable for the reversible labeling of amino groups [5,6]. Therefore, the DAM reagent allows the reversible fluorescence labeling of amines (Fig. 1), and is thought to be suitable for tracing and the recovery of a small amount of proteins and peptides in biological samples. In a previous study, we reported the new DAM reagent for the first time, and demonstrated the reversibility of labeling using benzylamine as a model compound of amines [4]. However, the precolumn and precapillary derivatization conditions had not been established. In addition, in the proteins and peptides, the characteristics of each amino group are thought to be different because of the different environment, and these compounds possibly lose their activities during the derivatization and regeneration reactions. Therefore, the application, which actually demonstrates the recovery of proteins, is needed to describe the usefulness of this new DAM reagent. In the present investigation, the precolumn and precapillary derivatization conditions have been investigated, and using insulin, catalase and lysozyme as the biologically active proteins, reversible fluorescence labeling with DAM is reported. Recovery of the biological activity has also been demonstrated.

2. Experimental

2.1. Materials

DAM was synthesized as described in our previous report [4]. Acetonitrile of HPLC grade, (trimethylsilyl)ethoxyacetylene and insulin (bovine) were obtained from Wako (Osaka, Japan). Benzylamine was from Nacalai Tesque (Kyoto, Japan). Catalase (*Aspergillus niger*), lysozyme (hen egg) and *Micrococcus luteus* cells were purchased from Seikagaku Kogyo (Tokyo, Japan). Water was purified using a Milli QII system (Millipore, Bedford, MA, USA). All other reagents and solvents were of reagent grade.

2.2. Derivatization of amino groups with DAM

DAM (3.8 mg) was suspended in acetonitrile (500 μl), and 10 μl (60 μmol) of (trimethylsilyl)-ethoxyacetylene was added. After heating at 60 °C for 90 min, 25 μl of the reaction mixture was added to 1 ml of the sample solution (6–500 μ*M* benzylamine and 50 μ*M* insulin). This reaction mixture was allowed to stand at 25 °C for 1 h, and a clear solution was obtained. For the derivatization of catalase (1 mg/ml) and lysozyme (1 mg/ml), DAM (5.7 mg) was suspended and 15 μl of (trimethylsilyl)ethoxyacetylene was added, then 100 μl of the reaction mixture was used for the labeling reaction.

2.3. Regeneration of insulin, catalase and lysozyme

To the derivatization reaction mixture (1 ml) of insulin, catalase and lysozyme, 50 μl of 1 or 2 *M* phosphoric acid was added. The pH of these regeneration reaction mixtures was 2.5–2.8 and 1.5–1.8,

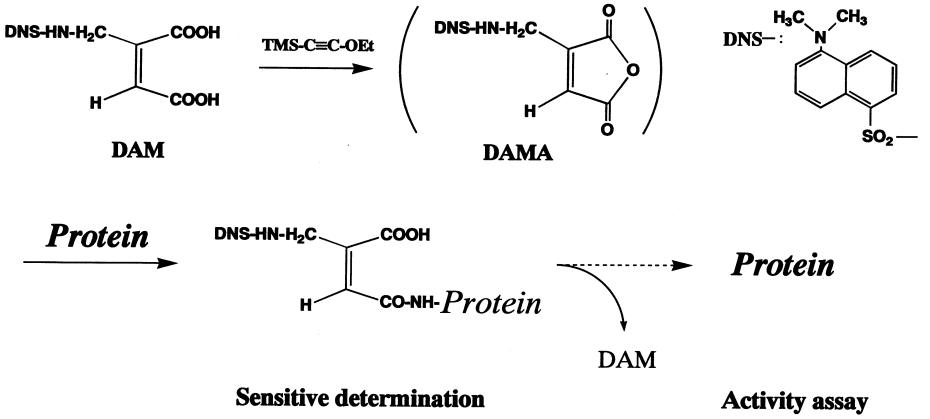

Fig. 1. Reversible fluorescence labeling of proteins with DAM.

respectively. The reaction mixture was then stored at 25 or 40 °C to regenerate the proteins.

2.4. HPCE system

An HPCE system was used to monitor the derivatization and regeneration of benzylamine, insulin and catalase. The system consisted of an 890-CE (Jasco, Tokyo, Japan), an 870-CE detector (Jasco) and a Chromatocorder 21 (SIC, Tokyo, Japan). A fused-silica capillary of 50 μm I.D.×65 cm (effective length=45 cm) was used. The running buffer was 25 m*M* borate–25 m*M* phosphate–NaOH (pH 8.0), and electrophoresis was carried out at a 20 kV constant voltage. Sampling was electrophoretically operated at 20 kV for 2 s. The absorbance was monitored at 210 nm. For the investigation of the precapillary derivatization of benzylamine with DAM, the electrophoresis and sampling were carried out at 15 kV.

2.5. HPLC system

An HPLC system was used to monitor the derivatization and regeneration of the lysozyme. The system consisted of a PU-980 pump (Jasco), a 7725I injector (Rheodyne, Cotati, CA, USA), an FP-920S fluorescence detector (Jasco), a UV-8010 detector (Tosoh, Tokyo, Japan), and an 807-IT integrator (Jasco). The analytical column used was a Super-Octyl (4.6 mm I.D.×100 mm, Tosoh) maintained at 40 °C in a CO-965 column oven (Jasco), and the mobile phase was an aqueous acetonitrile solution (20–50% (v/v), linear gradient from 0 to 30 min) containing 0.1% (v/v) HCl. The flow rate was 1.0 ml/min. Both the absorbance at 280 nm and the fluorescence at 540 nm with excitation at 325 nm were monitored.

2.6. MALDI–TOF-MS

A Perceptive Biosystem Voyager™ (Elite, Foster City, CA, USA) was used to determine the molecular masses of the derivatives and regenerated insulin and lysozyme. Insulin and the lysozyme solution (2 μl) were mixed with 18 μl of the matrix solution (saturated sinapinic acid solution in aqueous 33% acetonitrile 0.007% TFA (v/v)) [7]. One microliter was subjected to analysis.

2.7. Enzyme activity assay of catalase

Catalase solution (25 μl) was added to 475 μl of the 50 m*M* potassium phosphate buffer (pH 7.0), and 100 μl of the solution was added to 1.9 ml of H_2O. One milliliter of 60 m*M* H_2O_2 in 50 m*M* potassium phosphate buffer (pH 7.0) was then added and the absorbance at 240 nm was monitored at 37 °C for 10 min [8].

2.8. Enzyme activity assay of lysozyme

The lysozyme solution (25 μl) was added to 475 μl of the 100 m*M* potassium phosphate buffer (pH 6.2), and 100 μl of the solution was added to 2.9 ml of *M. luteus* cells suspended (0.2 mg/ml) in 100 m*M* potassium phosphate buffer (pH 6.2). The absorbance at 540 nm was then monitored at 37 °C for 10 min [9].

3. Results and discussion

3.1. Precolumn or precapillary derivatization of amino groups with DAM

DAM is dehydrated in the presence of (trimethylsilyl)ethoxyacetylene, an anhydrous reagent, and reacts with an amino group via its anhydride. The reaction conditions for the precolumn or precapillary derivatization have been established in the present investigation. To estimate the amount of DAM anhydride formed, benzylamine, a model compound having an amino group, was derivatized with DAM and the resultant derivative (BDAM, *N*-benzyldansylaminomethylmaleamic acid) was determined using an HPCE system. According to the structure of DAM, two positional isomers of BDAM were produced. These two isomers have the same electrophoretic mobility under the present HPCE conditions, therefore, the total amount of two isomers of BDAM were determined as the derivative. The peak height of BDAM becomes higher with an increase in the (trimethylsilyl)ethoxyacetylene content up to 60 μmol and does not change much at higher contents.

The effects of the reaction temperature and reaction time of the dehydration have also been examined. Concerning the reaction temperature, the highest BDAM peak was observed at 60 °C. As for the reaction time, a large peak of BDAM was observed from 60 to 90 min and gradually decreased thereafter. As a result, 60 μmol of (trimethylsilyl)-ethoxyacetylene was added and heated at 60 °C for 90 min for the dehydration of DAM, and the reaction mixture was used for the derivatization of the amino groups. Linearity of the calibration curve and day-to-day precision have been investigated using benzylamine as a model amino-compound. The amount of BDAM formed by the derivatization increased with an increase in the amount of benzylamine used, and a linear regression line ($r>$ 0.999) was observed from 6 to 200 μ*M* of benzylamine. Day-to-day precision of the BDAM amount formed has been examined using 50 μ*M* of benzylamine for 4 days and the RSD obtained was 3.3%. Under these conditions, benzylamine was completely derivatized with DAM and no peak of benzylamine was observed after the derivatization. These results indicate that the derivatization reaction of amino groups with DAM could be quantitatively performed with sufficient reproducibility by the present procedure. As we reported previously [4], the fluorescence detection limit of BDAM is 100 fmol, and benzylamine was completely regenerated under mild acidic conditions, indicating that DAM is a suitable reagent for reversible fluorescence labeling of amino groups.

3.2. Reversible derivatization of insulin

The reversible derivatization of insulin has also been investigated. Insulin (bovine, M_r=5733) was derivatized with DAM and was regenerated in the acidic media. The derivatization and regeneration reactions were monitored using an HPCE system and a MALDI–TOF-MS system. Fig. 2 shows the electropherograms of the derivatized insulin (a) and regenerated insulin (b, 24 h; c, 144 h). In the reaction mixture of the derivatized insulin, the insulin peak was not observed and several peaks (mainly four peaks) of DAM-derivatized insulin were observed (Fig. 2(a)). Because insulin has three amino groups, several derivatives caused by multiple labeling are likely to be observed [10]. In the regeneration reaction mixture, the amount of insulin increased with the increase in the reaction period, and the peaks of the DAM-derivatized insulin disappeared except for one peak (Fig. 2(c)). To investigate the molecular mass of the derivatives, as well as to demonstrate the regeneration of the native insulin, the reaction mixtures of the derivatized and regenerated insulin were also analyzed using the MALDI–TOF-MS system. After derivatization of the insulin

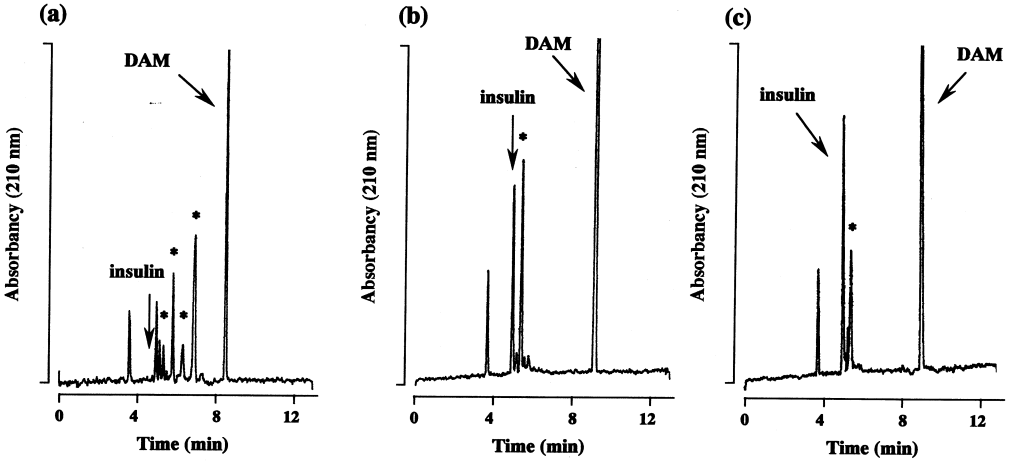

Fig. 2. Electropherograms of derivatized insulin with DAM (a), and regenerated insulin (b,c). Regeneration was carried out at pH 1.8 and 25 °C for 24 h (b), and for 144 h (c). The asterisks indicate the peaks produced by the reaction of insulin with DAM. The other conditions are described in the text.

with DAM, the mono-, di- and tri-labeled insulins were observed. The molecular masses obtained for these peaks were 6095, 6457 and 6816, respectively (insulin=5733, DAM=360). The peaks of the di- and tri-labeled insulin disappeared after the regeneration reaction, and the peaks of the native insulin and mono-labeled insulin were observed. Insulin has an amino group in the A chain (^{1}Gly) and also has two amino groups in the B chain (^{1}Phe, ^{29}Lys), therefore, these three amino groups might be derivatized with DAM. Under acidic conditions, the native insulin was then successfully regenerated. The time course of the insulin regeneration has been investigated under various conditions. The amount of regenerated insulin increased by lowering the pH of the regeneration reaction mixture and by increasing the regeneration period, and 60% of the initial amount was observed at pH 1.8 and at 25 °C after a 144-h regeneration. Concerning the reaction temperature, insulin was rapidly regenerated at a high temperature and the same amount of insulin as that regenerated at 25 °C for 144 h was observed after a 24-h regeneration at 40 °C.

3.3. Reversible derivatization of catalase

Catalase is an enzyme which converts hydrogen peroxide into water and oxygen. Catalase from *A. niger* (240 kDa) was derivatized with DAM and regenerated in the acidic media. The derivatization and regeneration reactions were monitored using an HPCE system. Throughout the reaction, the enzymatic activity was also measured using the change in absorbance at 240 nm caused by the degradation of hydrogen peroxide. Fig. 3 shows the electropherograms of catalase before (a) and after (b) the derivatization with DAM. The electropherogram obtained after the regeneration in the acidic media is also shown in Fig. 3(c). As shown in Fig. 3(a), the migration time of the native catalase is about 4.0 min. However, a broad peak was observed at 5–8 min after the derivatization reaction. The catalase has 84 amino groups [11]. Therefore, numerous derivatives might be produced due to the multiple labeling, which could not be simultaneously separated by the HPCE system, resulting in the observation of a broad new peak. The peak was gradually changed with the increase in the regeneration reaction time, and almost the same peak as the native catalase was observed after a 24-h regeneration at pH 2.5 and 25 °C. The enzymatic activity of the catalase did not significantly change by either the derivatization or regeneration, and the derivatives as well as the regenerated catalase have almost the same activity as the native enzyme. These results indicate that the derivatization and regeneration reactions have been sufficiently

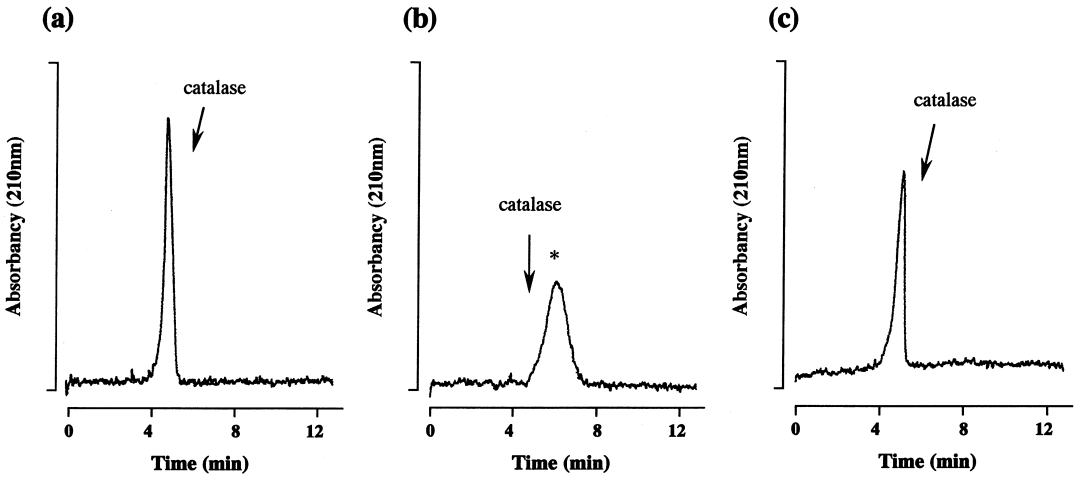

Fig. 3. Electropherograms of catalase obtained before (a) and after (b) the derivatization with DAM, and obtained after the regeneration of the derivatized catalase in the acidic media (c). The arrows indicate the migration time of the native catalase. The asterisk indicates the peak produced by the reaction of catalase with DAM. The other conditions are described in the text.

accomplished under mild conditions for the catalase to maintain the enzymatic activity.

3.4. Reversible derivatization of lysozyme

The reversible derivatization of the lysozyme (hen egg, M_r=14 307) was investigated. The derivatization and regeneration were monitored using an HPLC system and a MALDI–TOF-MS system. Fig. 4 shows the chromatograms of the lysozyme before (a) and after the derivatization reaction (b) and after the regeneration in the acidic media (c). Each peak was detected by the absorbance at 280 nm. The native lysozyme, which was eluted at the retention time of about 15 min (Fig. 4(a)), was not observed after the derivatization and the peaks of the DAM-derivatized lysozyme were observed (Fig. 4(b)). Fluorescence detection at 540 nm was also carried out with excitation at 325 nm, and a strong fluorescence was observed at the retention time of the DAM-derivatized lysozyme. The molecular masses of the derivatized and regenerated lysozyme were also monitored by the MALDI–TOF-MS system. Fig. 5 shows the mass spectra of the native lysozyme (a), derivatized lysozyme (b) and regenerated lysozyme (c). In the derivatization reaction mixture, the native lysozyme was not observed and the peaks of the DAM-derivatized lysozyme were observed (Fig. 5(b)). The molecular masses of these peaks are 15 401, 15 756 and 16 117, therefore, 3–5 amino groups of the lysozyme are labeled with DAM. The native lysozyme was then regenerated from the derivatives under mild acidic conditions at pH 2.5 and 40 °C. In the regeneration reaction mixture, the peaks of the DAM-derivatized lysozyme completely disappeared and the peak of the native lysozyme was observed (Fig. 5(c)). The enzymatic activity was also monitored by the degradation of the *M. luteus* cells by measuring the absorbance at 540 nm. The time course is shown in Fig. 6. After the derivatization with DAM, no lysozyme activity was observed in the reaction mixture. However, the bacteriolytic activity increased with the increased regeneration period, and 95% of the activity for the native lysozyme was observed after a 48-h regeneration (pH 2.5, 40 °C). Lysozyme has seven amino groups, and all of them are exposed to the enzyme surface [12]. The bacteriolytic activity of the lysozyme was reported to be

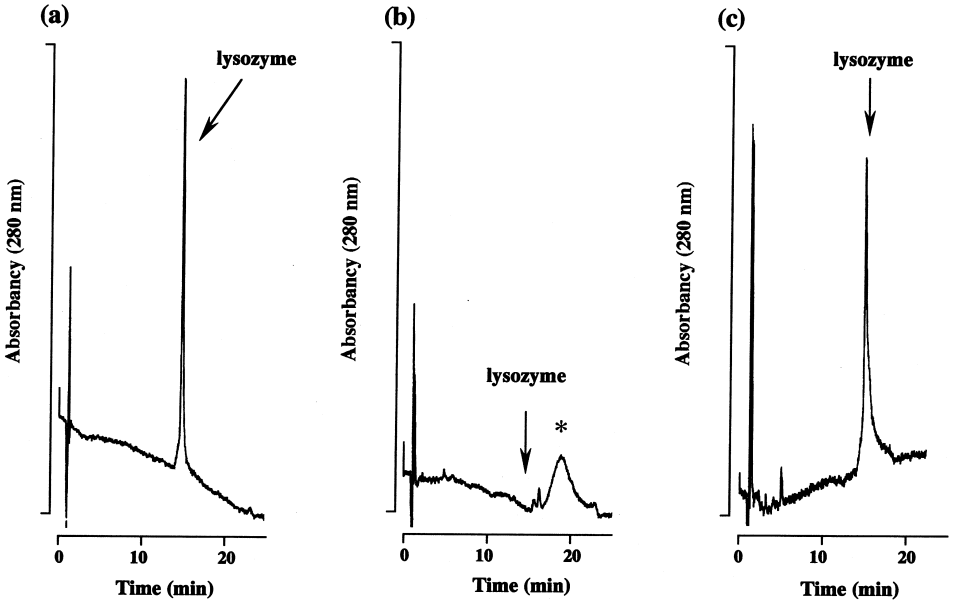

Fig. 4. Chromatograms of lysozyme (a), derivatized lysozyme with DAM (b), and regenerated lysozyme (c). Regeneration was carried out at pH 2.5 and 40 °C for 48 h. The arrows indicate the migration time of the native lysozyme. The asterisk indicates the peak produced by the reaction of lysozyme with DAM. The other conditions are described in the text.

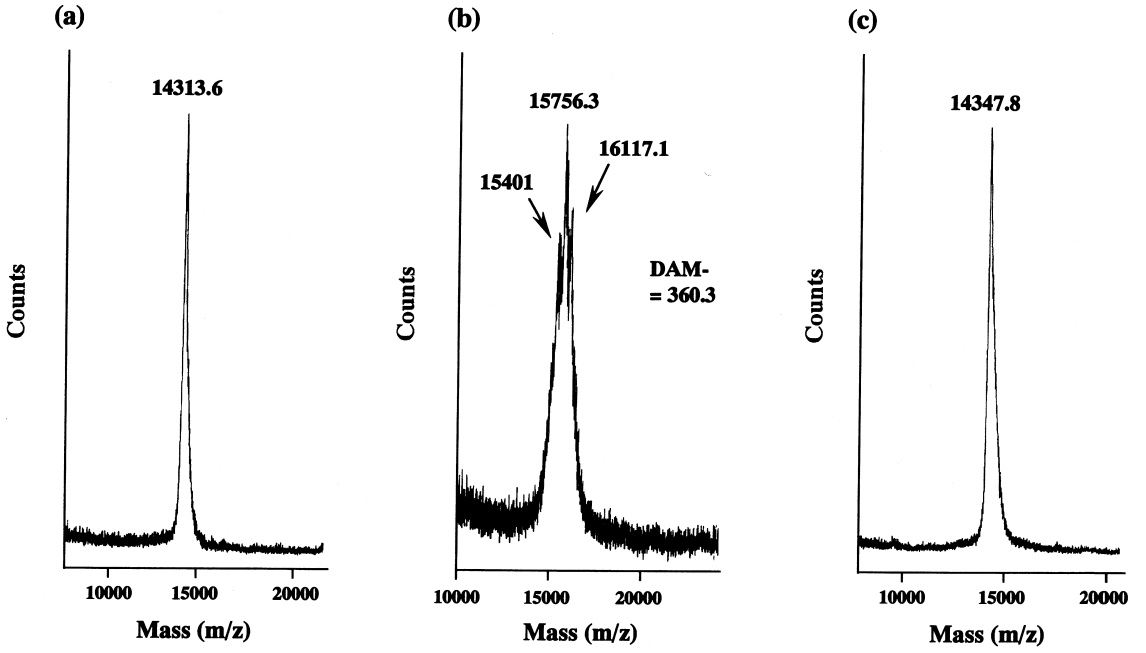

Fig. 5. Mass spectra of lysozyme (a), derivatized lysozyme with DAM (b), and regenerated lysozyme (c). Regeneration was carried out at pH 2.5 and 40 °C for 48 h. The other conditions are described in the text.

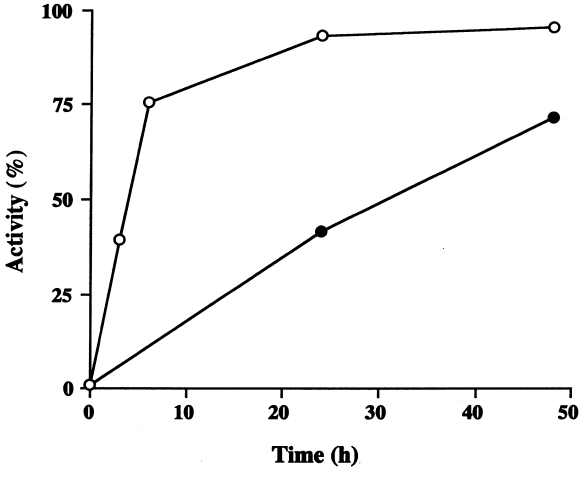

Fig. 6. Time course of bacteriolytic activity of lysozyme observed during the regeneration reaction in the acidic media. Regeneration was carried out at pH 2.5. Open circles indicate the values obtained by the regeneration at 40 °C, and the closed circles are those obtained at 25 °C. The other conditions are described in the text.

reduced by the acetylation of these amino groups [12]. Therefore, it is likely that the bacteriolytic activity has not been observed after the derivatization with DAM, then the activity increases by regeneration of the native lysozyme. These results indicate that the present procedure is also mild enough for the lysozyme.

In the present investigation, reversible fluorescence labeling of proteins has been demonstrated. In the derivatizing reaction mixture of insulin and lysozyme, several derivatives were observed due to the multiple labeling of proteins; which should be improved further, because the control of labeling reaction [13] is important for sensitive detection of proteins. In the next step, the regeneration of proteins was performed under acidic conditions, and approximately the same activity as the native protein has been observed after the regeneration for both catalase and lysozyme. Although this method has some limitations that not all of the proteins are stable under acidic conditions, the results obtained in the present investigation indicate that this method is suitable for the reversible fluorescence labeling of biologically active substances.

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