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Original Article

Studies on chemical modification of papain by 5-chlorosulfonyl-2-oxobenzimidazole as biotin model compound

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Summary 5-chlorosulfonyl-2-oxobenzimidazole(**1**) was synthesized. On adding **1** to the suspension of papain in acetonitrile containing formamide, **1** was introduced into the papain in a yield of 7%, suggesting that **1** modified papain chemically to give 2-oxobenzimidazolesulfonyl papain(OBI- papain). Also, it was found interestingly that papain activity of OBI-papain was maintained and that SH group in the active center in the large cleft of papain was free. Accordingly, It expects that OBI-papain might have other catalytic action except papain activity.
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Keywords : 5-chlorosulfonyl-2-oxobenzimidazole, papain, biotin model compound, artificial enzyme

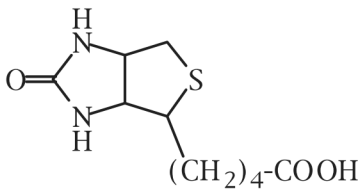
Introduction

The author has studied the production of the artificial enzymes utilizing the coenzyme model compounds, and reported the investigation of the introduction of vitamin B_6 model compound into apo glutamate oxalacetate transaminase (apo GOT).[1, 2)] As well as vitamin B_6, biotin, one of coenzymes, has turned out to work in a unique way.

Biotin contains 2-oxoimidazole ring active catalytically as the general acid-base, and catalyze the biochemical reactions such as carboxylation, transcarboxylation, and decarboxylation.

Therefore, it is expected that to introduce the imidazolone ring into various proteins and other things such as organic and inorganic supports will lead to a of creation of the new artificial enzymes. However, biotin does not have the reactive group to bind covalently.

As the biotin model compound containing



2-oxoimidazole ring and the chemically modifying group, we synthesized 5-chlorosulfonyl-2-oxobenzimidazole (**1**) including the functional group to bind covalently, and investigated the chemical modification of proteins with **1**.

Papain, one of proteins, has a large cleft into which one coenzyme such as biotin can be taken,[3)] and furthermore has a SH group which can react easily with **1**. Therefore, papain was selected for the support of **1**.

This paper reports the synthesis of **1** and the results of experiments of the introduction of **1** into papain.

Experimental

Materials; 2-oxobenzimidazole and chlorosulfonic acid were purchased from Wako Chemicals Co., Ltd. Twice-crystallized and lyophylyzed papain was commercialized from Sigma–Aldrich Corporation.

Equipments: FT-NMR spectrometer(DPX400S, BRUKER, HITACHI High-Technology Ltd.), CHN-corder (MT-3, YANACO Corporation), and spectrophotometer (UV-2000, HITACHI High-Technology Ltd.) were used.

Preparation of **1** : By improving the methods reported earlier,[1, 2)] the direct chlorosulfonylation of benzene ring was carried out as follows. Into 20ml of

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conc. sulfuric acid cooled at -15～-20℃, 1.0g of 2-oxobenzimidazole was added stepwise. Into the mixture, 10ml of chlorosulfonic acid was added dropwise under stirring, and the reaction mixture was heated at 60-65℃ for 4h. After cooling, the reaction mixture was poured onto 300g of ice dropwise. The solid released was collected by filtration and dried. Repeating the recrystallization from acetone-n-hexane gave 1.32g of the colorless prisms. Yield, 76.4%. d.p., 233.5-235℃. Anal. Calcd for $C_7H_5N_2O_3SCl$: C, 36.13; H, 2.15; N, 12.04. Found: C, 36.24; H, 2.21; N, 11.67. ^{1}H-nmr (DMSO, 400MHz);δ 10.69 (s, 1H), δ 10.65 (s, 1H), δ 7.23 (dd, J=1.5 & 8.0 Hz, 1H), δ 7.18 (d, J=1.5 Hz, 1H), δ 6.85 (d, J=8.0Hz, 1H). Beilstein reaction and sodium nitroprusside reaction of **1** were positive, supporting the existence of Cl and S atom, respectively. **1** is not soluble in water, slightly soluble in hexane, and soluble in acetone, chloroform, and acetonitrile.

***Treatment of Papain with* 1** : Into 10ml of anhydrous acetonitrile contai-ning 0 — 0.3ml of formamide, 50mg of papain was suspended. The papain suspension was stirred for 1 h at room temperature in the presence of N_2 gas. Into the suspension 4mg of **1** was added, and the suspension was stirred for 24 h at room temperature in the presence of N_2 gas. By filtration, the modified papain, 2-oxobenzimidazole-5-sulfonyl papain (OBI-papain), was collected. To remove the excess of **1** and sulfonate of **1**, 0.02M-phosphate buffer solution (pH7.4) of OBI-papain was dialyzed against the same buffer solution as above at 5℃ for 30h.

Measurements of the Papain Activities : Papain activities were measured by the method of Bradford.[4)]

***Spectroscopy of* 1, *Native Papain, and the Papain Treated with* 1** : **1**, the native papain, and OBI-papains were dissolved in the above buffer solution and carried out to the spectroscopy in uv area, followed to estimate the ratios of OBI group in OBI-papains.

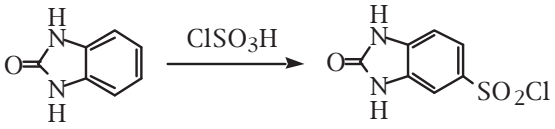
Determinations of Protein Concentrations of OBI-Papains : OBI-papains dissolved in the same buffer solution as above were carried out to determine the protein concentrations by Bradford method using bovine serum albumin as the standard.[5)]

Titrations of SH Groups in Papains : The number of SH groups in papains were estimated by the titration method using Ellman's reagent.[6)]

***Denaturations of Papains by* Cu^{2+}** : Papain activities of OBI-papains in the case of adding a trace of Cu^{2+} were also tested.

Results and Discussion

***Preparation of* 1** : 2-oxobenzimidazolone was utilized for the biotin model compound, for a benzene ring is favorable to electronic effects and easy to be substituted by the chemical modifier. So, the direct chlorosulfonylation of 2-oxobenzimidazole was carried out by the methods of the previous reports.[1, 3)] From the data in NMR spectra of **1**, the position of chlorosulfonyl group on benzene was determined.



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Scheme 1

***Properties of* 1** : Chlorosulfonyl group of **1** reacts easily with basic groups. In spite of being not soluble in water, **1** is dissolved into water gradually by hydrolysis of chlorosulfonyl group after 6h at room temperature, which means it is much easier to be hydrolyzed in alkali side within 1h, even if at 5℃. Being dissolved in buffer solution (0.02M-phosphate, pH7.4), **1** was hydrolyzed within 2h at room temperature. Therefore, to react **1** with papain, it is not suitable to dissolve papain into water.

Sulfonate of **1** obtained by hydrolysis of **1** showed no papain activity in both of water and acetonirile.

***Treatment of Papain with* 1**: Although modifications of enzymes are usually carried out in water, organic solvents were preferable, considering the instability of **1** in water. After testing hydrophilic organic solvents except alcohols to react **1** with papain, referring to the studies of the influences of the organic solvents on the proteins,[7, 8)] anhydrous acetonitrile containing formamide, effective on unfolding the protein, was used. As expected, the quantity of OBI introduced into papain by **1** increased with increment of formamide. The results of these were described in Table 1.

Thus, in this investigation, it was made clear that **1** could be utilized as the chemical modifier to protein in the organic solvent system.

Spectroscopy of* 1, *Native Papain, and the OBI-Papains : By the spectroscopy, it was found that **1** had distinct two peaks 241nm and 283nm, as shown in Fig. 1. This spectral pattern was the same to that of OBI.

Native papain showed the same pattern as protein in spectra including a peak at 278nm. Papain also, treated with **1** in acetonitrile not containing formamide

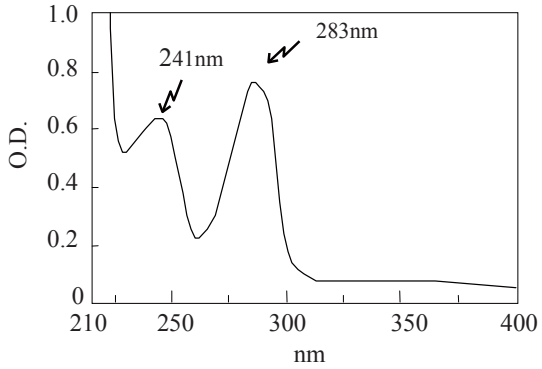

Fig. 1. Spectral pattern of **1**. **1** in buffer solution of 0.05M-phosphate (pH7.4), after hydrolysis in the same buffer solution at 18℃ for 2h. **1**, as similar to OBI, shows 2 peaks at 241nm and 283nm, respectively.

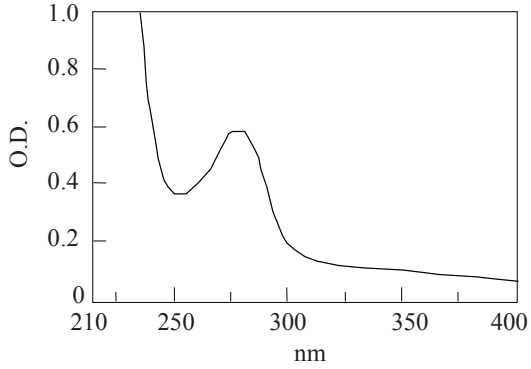

Fig. 2. Spectral pattern of OBI-papain. OBI-papain in the same buffer solution as that in **Experimantal**, after treatment with **1** and dialysis against the same buffer solution at 18 for 2h.

and dialyzed against 0.02M- phosphate buffer solution (pH7.4) 5℃ for 30h , showed the same pattern as native papain in spectra..

In the case of OBI-papain obtained in the condition of 1% of formamide, the shoulder at 241nm by the absorption of **1** appeared clearly, as Shown in Fig.2.

Measurements of the Papain Activities : By harming papain activities by the organic solvents, native papain activities decreased a little by acetonitrile and formamide. At the same time, OBI-papains maintained the papain activity. The above results are shown in Table 1.

Inactivations of Papains by Cu^{2+} : OBI-papains were inactivated by adding Cu^{2+}, as well as native papain.

Estimation of the Ratios of* 1 *and Papain in OBI-papains : Many of spectral data obtained by the above spectroscopies were utilized for the estimations of ratios of **1** and papain in OBI-papains.

In case of OBI-papain modified by **1** in the condition of 1% of formamide, the absorbance at 241nm and 283nm were 0.198 and 0.234, respectively.

From the other data obtained by the above spectroscopy, the following values and equations were utilized.

The process for estimation is as follows:

First ; molar extinction coefficients at 241nm and 283nm of **1** were $2.76\times10^3 cm^{-1}mol^{-1}$ and $3.45\times10^3 cm^{-1}mol^{-1}$, respectively.

Second; molecular extinction coefficients of the at 241nm and 283nm of native papain were $52.3\times10^3 cm^{-1}mol^{-1}$ and $54.1\times10^3 cm^{-1}mol^{-1}$, respectively.

Third; when each of the concentration of OBI and papain in OBI-papain will be $\boldsymbol{x}$ mol/l and $\boldsymbol{y}$ mol/l respectively, the following equations will be given.

$$2.76\times10^3\times\boldsymbol{x} + 52.3\times10^3\times\boldsymbol{y} = 0.198$$
$$3.45\times10^3\times\boldsymbol{x} + 54.1\times10^3\times\boldsymbol{y} = 0.234$$

Accordingly, the ratio of OBI/papain in this case was estimated to be 0.05/1. The ratios in other cases estimated are described in Table 1.

Table 1. Relations of concentrations of formamide with ratios of OBI group and relative activities of papains.

papain	organic solvent[a)]	molar ratio of OBI in OBI-papain	relative activity of papain[b)]
native	non	−	1
	MeCN[c)]	−	0.95
	MeCN + 1% of FA[d)]	−	0.92
OBI-	MeCN	0.00	0.92
	MeCN + 0.1% of FA	0.04	0.98
	MeCN + 1% of FA	0.07	0.91
	MeCN + 3% of FA	0.06	1.05

a) For native papain, to examine the damage by solvent used to modification. For OBI-papain, **1** was contained. b) The activity of native papain, not treated with organic solvent, was taken as 1. c) MeCN is acetonitrile. d) FA is formamide. Details are described in **Experimental**.

When formamide was not contained, chemical modification didn't occur. It seems that it is necessary to unfold the texture of papain by formamide for the chemical modification. However, when 5% or more of formamide was added, the papain activity largely decreased.

Interestingly, OBI-papains was found to maintain the papain activity, as shown in the Table 1. On the other hand, OBI-papains was inactivated by adding Cu^{2+}. Furthermore, titration experiments for SH group showed that SH group was free. These facts suggest that the SH group, which is very reactive with **1**, stays to be not modified by **1** in the active site of OBI-papain. Accordingly, it is thought that the large cleft containing the active site was not opened in spite of the use of formamide.

As shown in Table 1, however, it was also the fact that a small quantity, 7%, of OBI groups were introduced into the papain. Therefore, **1** reacted with the reactive nucleophilic group in somewhere without [other than] the cleft.

In this study on OBI-papain, different enzyme activity from papain was not observed. Further studies are waited to shed light on new enzyme activities of OBI-papain to make the combined enzyme.

Biotin has high affinity to avidin, which suggests that OBI-papain may be utilized as something concerning avidin.

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ビオチンのモデル化合物としての5-クロロスルホニル-2-オキソベンズイミダゾールによるパパインの化学修飾に関する研究

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要　旨

ビオチンモデル化合物として5-クロロスルホニル-2-オキソ-ベンズイミダゾール（**1**）を合成した。ホルムアミド含有アセトニトリルのパパイン懸濁液に**1**を作用させると，**1**が約７％の割合でパパインに導入された。その結果，**1**がパパインを化学修飾して2-オキソベンズイミダゾールスルホニルパパイン（OBI-パパイン）が生成したことが示唆された。興味あることに，OBI-パパインはパパイン活性を保持しており，またパパインの空洞部位にある活性中心のSH基も失われていなかったことなどから，OBI-パパインはパパイン活性以外の触媒作用を持っていることも期待される。