[Chem. Pharm. Bull. 16(12)2339—2342(1968)]

UDC 615.356 : 577.161.5

# The Reaction of 2-Methyl-1,4-naphthoquinone with Bovine Serum Albumin and Papain

Noboru Nakai and Jun-ichi Hase

*Faculty of Pharmaceutical Science, University of Toyama*[1]

(Received March 30, 1968)

1. 2-Methyl-1,4-naphthoquinone ($K_3$) reacted with sulfhydryl group of protein such as bovine serum albumin and papain to form a thioether linkage at 3-position of $K_3$ as well as sulfhydryl groups of low molecular compound.

2. $K_3$ bound to detectable sulfhydryl groups in the active site of native papain with high specificity and its binding was not cleaved by the addition of cysteine.

## Introduction

It has been previously reported that 2-methyl-1,4-naphthoquinone ($K_3$) reacted with sulfhydryl group of low molecular compound to form the thioether linkage at 3-position of $K_3$ with an absorption maximum at 420—430 mμ.[2]

The role of sulfhydryl group of papain was studied by several authors. Finkle and Smith reported that native papain contains less than 0.5 moles of sulfhydryl group in a molecule and that the activated enzyme by passage through a reducing column of bound thioglycollate contains 0.6 to 1.0 mole of sulfhydryl group.[3] Glazer and Smith also reported that the activated papain reduced with sodium borohydride contains one mole of sulfhydryl group in a molecule with the highest proteolytic activity.[4]

In the present study, we examined the interaction of $K_3$ with sulfhydryl group of bovine serum albumin and with that of papain.

### Materials and Methods

Bovine serum albumin (powder, fraction V) was purchased from Armour Laboratories, and crystalline papain was prepared from commercial dry papaya latex by the method of Kimmel and Smith.[5] 5,5′-Dithio-bis(2-nitrobenzoic acid) (DTNB) was synthesized from *m*-chlorotoluene as a starting material according to the modified method of Ellman.[6] Casein (Hammarsten) as a substrate of papain, was purchased from Wakō Pure Chemical Co.

Sulfhydryl group was spectrophotometrically determined with DTNB by the method of Ellman.[6]

Proteolytic activity of papain was assayed by the modified method of Kunitz[7] with the use of casein as its substrate.

The reaction of $K_3$ with bovine serum albumin was performed as follows. A mixture consisting of 80 μM $K_3$, 80 μM bovine serum albumin 0.1 M phosphate buffer (pH 6.5) and 10% ethanol (for the aid of dissolving $K_3$) was incubated at 0°. The progression of the reaction was assayed by measuring the characteristic absorption of thioether linkage at 3-position of $K_3$ at 430 mμ and by determining the decrease of sulfhydryl group.

The reaction of $K_3$ with papain was assayed in the following manner. $K_3$ dissolved in ethanol was preincubated with papain in 50 mM phosphate buffer (pH 7.0) at 30° for 30 min and then proteolytic ac-

1) Location: *3910, Gofuku, Toyama.*
2) N. Nakai and J. Hase, *Chem. Pharm. Bull.* (Tokyo), **16**, 2334 (1968).
3) B.J. Finkle and E.L. Smith, *J. Biol. Chem.*, **230**, 669 (1958).
4) A.N. Glazer and E.L. Smith, *J. Biol. Chem.*, **236**, 2948 (1961).
5) J.R. Kimmel and E.L. Smith, *J. Biol. Chem.*, **207**, 515 (1954).
6) G.L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).
7) K. Kunitz, *J. Gen. Physiol.*, **30**, 291 (1947).

tivity was assayed. Ethanol concentration in a mixture was less than 5% and each test was always compared with the control omitting $K_3$.

Papain concentration was determined spectrophotometrically by measuring at 278 mμ based on the absorbance of 2.50 $cm^{-1}$ $mg^{-1}$ of protein and a molecular weight of 21000.[4]

## Results

### The Reaction of $K_3$ with Bovine Serum Albumin

Fig. 1 shows the progressive reaction with time when $K_3$ was incubated with bovine serum albumin at 0° and at pH 6.5. Decrease in sulfhydryl groups was accompanied with increase in absorbance at 430 mμ. It required 24 hr to complete the reaction.

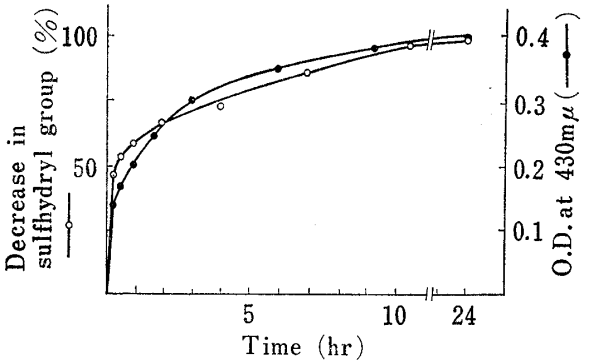


Fig. 1. Decrease in Sulfhydryl Group and Increase in Absorbance at 430 mμ by the Reaction of $K_3$ with Bovine Serum Albumin

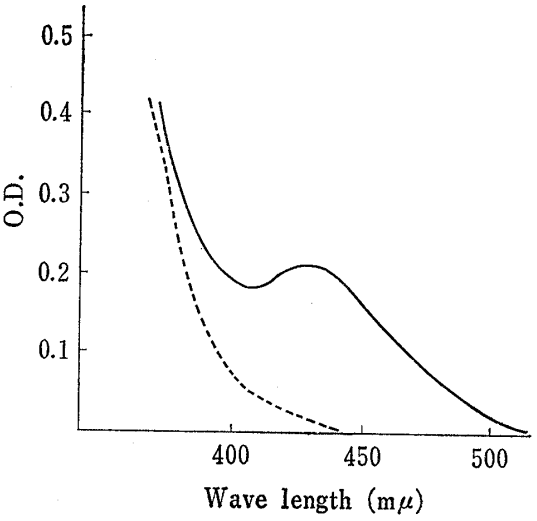


Fig. 2. Absorption Spectra

A mixture consisting of 40 μM $K_3$, 40 μM bovine serum albumin and 0.1 M phosphate buffer (pH 6.5) was allowed to stand for 24 hr at 0°.

——— in the presence of bovine serum albumin
– – – – – in the absence of bovine serum albumin

As can be seen in an absorption spectrum (Fig. 2), there was a markedly characteristic absorption maximum in the region of 420—430 mμ.

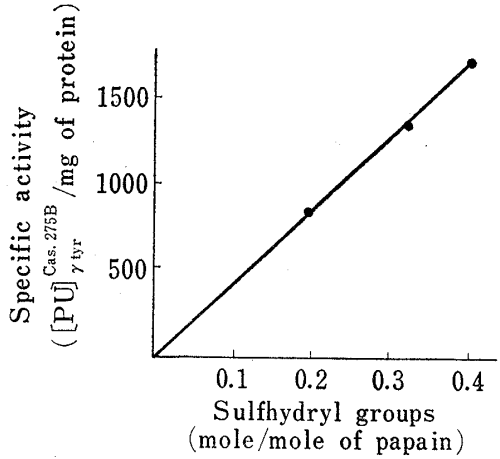


Fig. 3. Linear Correlation between Specific Activity and Sulfhydryl Content of Papain

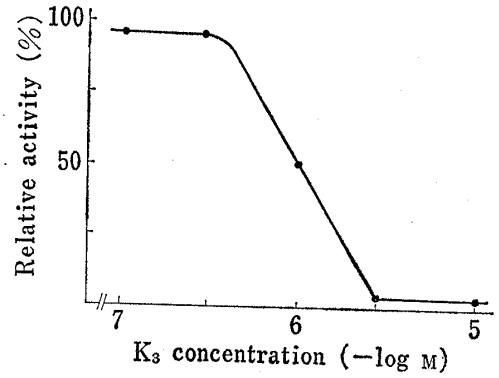


Fig. 4. Effect of $K_3$ Concentration on Papain Activity

Papain (1.6 μM) was preincubated with $K_3$ (0.1 μM to 10 μM) in 0.05 M phosphate buffer (pH 7.0) at 30° for 30 min.

From this result, it might be assumed that the sulfhydryl group of protein bound to form a thioether linkage at 3-position of $K_3$ in the same manner as that of low molecular compounds.

**The Interaction of $K_3$ with Papain**

Three preparations of crystalline papain which was obtained from dry papaya latex contained 0.2, 0.33 and 0.4 mole of sulfhydryl group per mole of the enzyme. Proteolytic activities of these preparations completely depended on the content of sulfhydryl groups (Fig. 3). $K_3$ powerfully inhibited proteolytic activity of papain.

The value of apparent $I_{50}$ for $K_3$ was found to be 1.0 μM under the condition at pH 7.0 and at 30° for 30 min of preincubation time (Fig. 4).

Native papain contains less than 0.5 mole of detectable sulfhydryl group per mole of the enzyme and is activated by the addition of reducing agents such as cysteine, thioglycolate and sodium borohydride.[3,4] An activated papain which contains one mole of detectable sulfhydryl group per mole of the enzyme, possesses the highest activity.[4]

In this study, native papain was completely inactivated by the incubation with $K_3$. $K_3$ inactivated papain was activated by the excess addition of cysteine. The activity was equal to the activated part of activity of the control (Omitting $K_3$ as is shown in Fig. 5).

This result suggests that the binding of $K_3$ with detectable sulfhydryl group in the active site of papain was not cleaved by the addition of cysteine and the apparent reactivation of $K_3$ inhibited enzyme is owing to exposure of the sulfhydryl group which was not measured by DTNB.

Effect of pH on the inactivation of papain with $K_3$ is shown in Fig. 6.

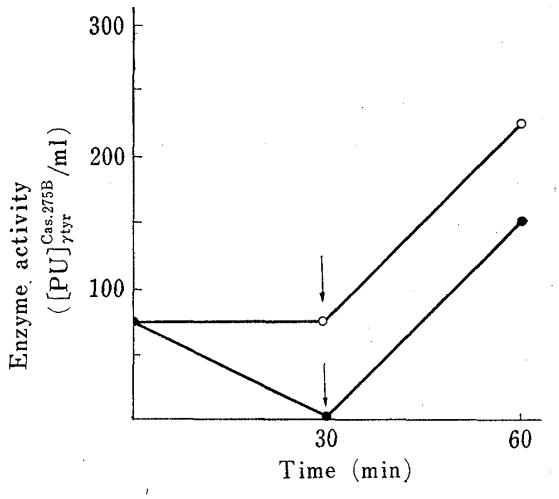


Fig. 5. Activation of $K_3$ Inhibited Papain by Cysteine

Papain (8.3 μM) was preincubated with $K_3$(—●— in the presence of 2.5 mM; —○— in the absence of) in 0.05 M phosphate buffer (pH 7.0). At the time as indicated by arrows, a mixture was added to the same buffer contained 0.04 M cysteine.

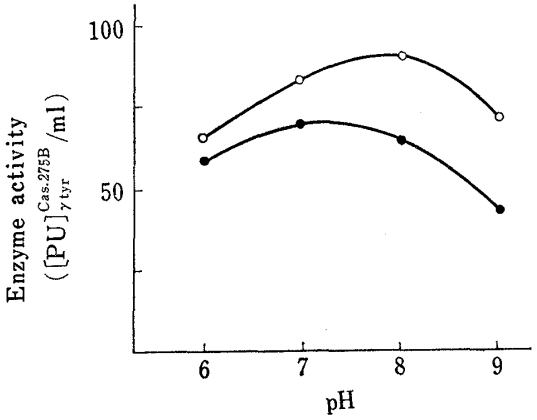


Fig. 6. Effect of pH on the Inhibition of $K_3$ for Proteolytic Activity of Papain

Papain (4.2 μM) was preincubated with $K_3$(—●— in the presence of 1.0 μM; —○— in the absence of) in 0.05 M phosphate buffer(pH 6.0 to 9.0) at 30° for 30 min and then was assayed.

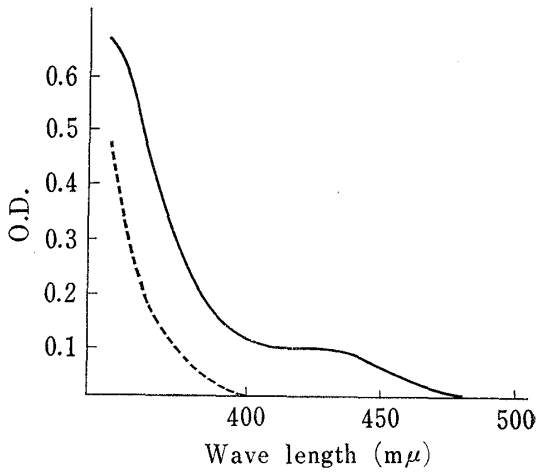


Fig. 7. Absorption Spectra

$K_3$ (2.5 μM) was preincubated with 2.3 μM papain in 0.05 M phosphate buffer (pH 7.0) at 30° for 30 min.

——— in the presence of papain
– – – – – in the absence of papain

Optimal pH of proteolytic activity of papain was about pH 8.0. However, the higher inactivation was observed in the more alkaline region. Thus, it seems probable that the hydrogen atom of 3-position of $K_3$ was activated in the more alkaline region.

The reaction mixture of $K_3$ and papain becomes yellow in color. As shown in Fig. 6, the absorption spectrum has a characteristic absorption band at about 430 mμ.

The results show that inactivation of papain by $K_3$ is attributable to the reaction of sulfhydryl group of active site of enzyme with $K_3$ to form a thioether linkage at 3-position.

## Discussion

It has been previously reported[2] that the thioether linkage at 3-position of $K_3$ has a characteristic absorption maximum at 430 mμ in its absorption spectrum. In the reaction of $K_3$ with bovine serum albumin, the rate of loss of sulfhydryl group of the protein was in parallel with the rate of increase in absorbance at 430 mμ and an absorption spectrum of a reaction mixture has a peak at 430 mμ.

This result shows that $K_3$ could be combined with sulfhydryl group of bovine serum albumin at 3–position of the former as well as with that of low molecular compound.

It was reported that native papain contains less than 0.5 mole of sulfhydryl group in the molecule when it was determined by spectrophotometric titration with N-ethylmaleimide and *p*-hydroxymercuribenzoate as well as by ampermetric titration,[4] and its enzyme activity is proportional to the number of reactive sulfhydryl group.[3,4] In addition, it was reported that native papain is fully reactivated by the reduction with cysteine, thioglycolate and sodium borohydride with which undetectable sulfhydryl group in the active site of papain may be exposed.

The present study confirmed the above results. Crystalline preparations of papain used contained 0.2—0.4 mole of sulfhydryl group per mole of the enzyme by measurement with DTNB.

An inactivated papain which was incubated with $K_3$, was activated by the addition of cysteine. This apparent activity was the same as that of native papain activated by the addition of cysteine.

The result indicates that $K_3$ reacted only with free sulfhydryl group but did not with undetectable sulfhydryl group in the active site of papain, accompanying with complete loss of enzyme activity, and that cysteine added could not cleave the binding of $K_3$ with sulfhydryl group of the protein and exposed undetectable sulfhydryl group in the active site.

**Acknowledgements** The authors are grateful to Mr. T. Akao and Miss T. Uehara for their technical assistance in this work.