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THE ENHANCEMENT OF THE ENZYMATIC ACTIVITY OF PAPAIN BY REACTION WITH *N*-BROMOSUCCINIMIDE*

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SUMMARY

1. Inactive papain (EC 3.4.4.10) has been reacted with *N*-bromosuccinimide at pH 3.5–5.5, *I* 0.1.

2. This treatment has resulted in an enzyme, which, upon activation and assay, has an enhanced *N*-benzoyl-DL-arginine *p*-nitroanilide-hydrolysing activity.

3. Maximum enhancement of activity, 250–300% over control value, was achieved at pH 4.5–5.5.

4. An enhanced activity, 100–150% over control value, persisted for at least 21 h at pH 4.5–5.5.

INTRODUCTION

The sulfhydryl-dependent protease, papain (EC 3.4.4.10), has been extensively investigated over the past several years. The complete amino acid sequence[1] has been determined and a tertiary structure postulated from X-ray studies[2]. From these data and from kinetic data[3] a picture has evolved for the mechanism of papain action. One of the amino acids presumed to be part of the active site is tryptophan[4,5]; *N*-bromosuccinimide has been suggested as a reagent with high specificity for modifying tryptophan residues in proteins[6,7] but may at the same time attack other amino acid residues[8]. This paper reports some results obtained by reacting inactive papain with *N*-bromosuccinimide, activating the modified protein with α-thioglycerol, and determining the *N*-benzoyl-DL-arginine *p*-nitroanilide (BAPA)-hydrolysing ability.

Inactive papain (Worthington Biochemical Corporation, 2 × crystallized), 200 mg, was dissolved in water to a final volume of 50.0 ml. The concentration of this solution was determined by measuring $A_{278\ m\mu}$ of a suitably diluted aliquot and using a value of $E^{1\%}_{1\ cm}$ of 25 (ref. 9) in 0.1 M acetate buffer (pH 5.0) and a mol. wt. of

Abbreviation; BAPA, *N*-benzoyl-DL-arginine *p*-nitroanilide.

* Portions of this work were presented at the FASEB meeting in Atlantic City, N.J., 1966.

22 600 (ref. 2)*. *N*-Bromosuccinimide (Araphoe Chemicals Inc.) solution was prepared daily just prior to use and stored at ice-water temperature. The solution was discarded and the experiment terminated at the appearance of any pink or orange color. The reaction of *N*-bromosuccinimide with papain was done as follows: a solution was prepared which contained 1.6 mg papain, 0.2 ml of a 0.5 *I* acetate buffer of the desired pH and water sufficient to bring the final volume to 1.0 ml and *I* 0.1 when the *N*-bromosuccinimide solution was added. To this solution of protein was added the required volume of a solution of *N*-bromosuccinimide which gave the desired *N*-bromosuccinimide: protein ratio. After the reaction had proceeded for the desired time aliquots of the reaction mixture were removed and the BAPA-hydrolysing activity was determined. For those experiments for which it was necessary to sample more than once for the BAPA-hydrolysing activity the following reaction mixture was prepared: 1.0 ml of a solution containing 8 mg of papain, 1.0 ml of 0.5 *I* buffer of the desired pH, 2.7 ml of water and 0.3 ml of 7 mM *N*-bromosuccinimide. This resulted in a *N*-bromosuccinimide:protein of 6:1. After the reaction had proceeded for the desired time aliquots were removed and assayed.

The activity assay using BAPA as substrate was done as follows[12]: an aliquot, 0.1 ml, of reaction mixture was added to an activation solution containing 2.3 ml 0.1 M acetate buffer (pH 5.0) and 0.1 ml activator solution. After 15 min 0.5 ml of BAPA solution was added and the reaction terminated after an additional 15 min by the addition of 0.5 ml of 0.3% iodoacetic acid solution. $A_{410\ m\mu}$ was read against a blank prepared by the addition of iodoacetic acid solution to the activation mixture prior to the addition of the BAPA solution.

The activator solution was prepared daily from 1 ml of 10% sodium EDTA solution in 0.1 M acetate buffer (pH 5.0) and 0.25 ml α-thioglycerol (Evans Chemetics) diluted to 5 ml with 0.1 M acetate buffer (pH 5.0). The BAPA solution was prepared by dissolving 50 mg BAPA·HCl (Mann Research Labs.) in 2.5 ml of dimethylsulfoxide. When the material was all dissolved, with gentle warming, the solution was diluted to 25 ml with 0.1 M acetate buffer (pH 5.0). The final assay solution contained $2.2 \cdot 10^{-6}$ M papain, $7.7 \cdot 10^{-4}$ M BAPA, $1.8 \cdot 10^{-3}$ M EDTA and $2.0 \cdot 10^{-3}$ M α-thioglycerol. During the 15 min of reaction the substrate was hydrolysed to the extent of 15% maximally using the enzyme preparation whose activity was enhanced most by reaction with *N*-bromosuccinimide. Usually less than 5% of the substrate was hydrolysed.

The data shown in Figs. 1 and 2 are typical for a set of experiments: the activity of mercaptan activated *N*-bromosuccinimide-modified inactive papain was greater than activated unmodified papain.

The BAPA-hydrolysing activity of 15-min *N*-bromosuccinimide-modified papain is shown in Fig. 1. The activity of unmodified papain was taken as 100%. At all the pH values at which the reaction took place the activity reached a maximum when 6 moles of *N*-bromosuccinimide were reacted with 1 mole of papain. Even at high *N*-bromosuccinimide:papain ratio of 20:1 at pH's 5.0 and 5.5 there was still a doubling of activity.

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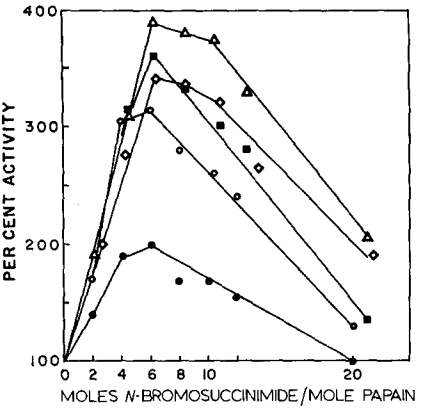
* A more correct mol. wt. is 23 405. This is obtained by subtracting one from the value of 23 406 (ref. 10) because the amino acid at position 64 is not aspartic acid but is asparagine[11]. This change is insufficient to change the final results reported here.



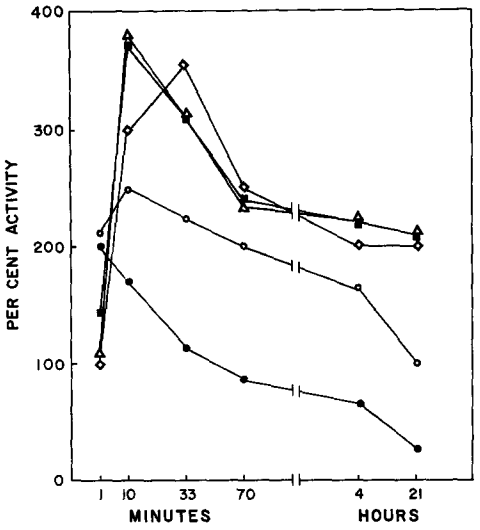


Fig. 1. The change in BAPA-hydrolysing activity caused by different amounts of *N*-bromosuccinimide reacting for 15 min with inactive papain and then activating. Reaction with *N*-bromosuccinimide at pH 3.5 (●), pH 4.0 (○), pH 4.5 (■), pH 5.0 (△), pH 5.5 (◇). The ordinate, percent activity, was calculated by dividing the $A_{410\ m\mu}$ produced by activated *N*-bromosuccinimide-treated papain hydrolysing BAPA for 15 min by the $A_{410\ m\mu}$ produced by activated unmodified papain hydrolysing BAPA for 15 min multiplied by 100, *i.e.* $[A^{NBS}_{410\ m\mu}/A_{410\ m\mu}] \times 100$.

Fig. 2. The change in BAPA-hydrolysing activity caused by *N*-bromosuccinimide reacting for different lengths of time with inactive papain; *N*-bromosuccinimide:papain = 6 moles/mole. The abscissa is the length of time the inactive papain has reacted with *N*-bromosuccinimide before assay. Reaction with *N*-bromosuccinimide at pH 3.5 (●), pH 4.0 (○), pH 4.5 (■), pH 5.0 (△), pH 5.5 (◇). The ordinate, percent activity, was calculated by dividing the $A_{410\ m\mu}$ produced by activated *N*-bromosuccinimide-treated papain hydrolysing BAPA for 15 min by the $A_{410\ m\mu}$ produced by activated unmodified papain hydrolysing BAPA for 15 min multiplied by 100, *i.e.* $[A^{NBS}_{410\ m\mu}/A_{410\ m\mu}] \times 100$.

The activities of papain preparations which had been allowed to react with *N*-bromosuccinimide for varying periods of time at different pH values are shown in Fig. 2. The conditions of the reactions were such that the papain: *N*-bromosuccinimide ratio was 1:6. At pH 3.5 the activity decreased continuously after having reached a maximum sometime prior to 1 min of reaction with *N*-bromosuccinimide. Within 50 min the activity had fallen to the control value and after 21 h the activity was about one-quarter of the control. At pH 4.0 after 1 min of reaction with *N*-bromosuccinimide the activity had doubled and after 4 h the activity was still one and one-half times the control value. Only after 21 h was the control value reached. At pH's 4.5, 5.0 and 5.5 fairly similar results were seen. After 10 min reaction with *N*-bromosuccinimide the activity had tripled at pH 5.5 and almost quadrupled at pH's 4.5 and 5.0. After 33 min the activity at pH's 4.5 and 5.0 had decreased to about three times the control value while at pH 5.5 the activity had reached a maximum of three and one half times the control value. Commencing at 70 min and extending to 21 h the activities at pH's 4.5, 5.0 and 5.5 all followed the same pattern. At the termination of the experiment, 21 h, the activity was about twice the control values at these three pH values.

The usual result encountered with other enzymes treated with *N*-bromosuccinimide is complete and immediate inactivation[13]. *N*-Bromosuccinimide-modified papain behaved very differently, exhibiting an enhanced activity which persisted for some time at several of the pH values at which the modification occured*.

The increase in enzymatic activity after treatment with *N*-bromosuccinimide is not unique. At least two other enzymes showed a similar response. Dihydrofolate reductase[15] showed a two- to three-fold increase in activity with no change in tryptophan content but oxidation of sulfhydryl groups which resulted in disulfide bond formation. The increase in activity of Zn-alkaline phosphatase and Co-alkaline phosphatase was not due to tryptophan oxidation, which did occur, but to other causes[16].

Other laboratories have reported that *N*-bromosuccinimide reaction with papain[4] or mercuripapain[17] resulted in inactivation. One possible explanation for the inactivation of mercuripapain is that *N*-bromosuccinimide caused a loss of mercury with release of the essential sulfhydryl group which is oxidized by *N*-bromosuccinimide to a non-activatable state. No explanation is available to explain the results reported by SUN AND TSOU[4,18].

Papain modified by *N*-bromosuccinimide may prove to be useful for the preparation of an insoluble enzyme with more activity than is usually obtained in such preparations. When papain is insolubilized by being covalently bound to an insoluble support[19] or by cross-linking with aldehydes[20,21] the enzymatic activity is reduced. If *N*-bromosuccinimide-modified papain with an enhanced activity was insolubilized then the resulting insoluble papain, even after the expected reduction in activity, should be as active as normally activated soluble papain.

In the work described here we have used *N*-bromosuccinimide to modify inactive papain which can then be activated to produce an enzyme exhibiting enhanced activity. The causes of the enhancement of activatable activity are at present unknown**. There is some evidence that tryptophan residues are involved as a decrease in $A_{278\ m\mu}$ is seen[22]. However, until detailed studies of the kind done by KRONMAN *et al.*[8] on lysozyme can be done with papain no definite statement concerning the amino acids involved can be made.

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* Treatment of guanidinated papain with nitrous acid resulted in an immediate increase in activity[14].

** It is possible that what is being observed is not only due to a change in activatable papain by *N*-bromosuccinimide but also a conversion of so-called "irreversible" inactivated papain[23] to an activatable species. Thus the net effect of *N*-bromosuccinimide is to convert all the papain molecules to some form which when activated have a greater activity than found in a control not treated with *N*-bromosuccinimide.

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