ACTA CHEMICA SCANDINAVICA 14 (1960) 1439–1452

Short Communications

Myrosin-Catalyzed Formation of Turbidity and Hydrogen Sulfide from Sinigrin

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Recent investigations have demonstrated the formation of thiocyanate ion [1,2] and organic thiocyanates from S-glucosyl-thiohydroximyl sulfates (*iso*thiocyanate glucosides), separation of the sulfatase and thioglucosidase functions of myrosin [4], as well as the stimulation of the myrosin action by ascorbic acid [5]. These observations demonstrate that the fate of these thioglucosides can be enzymatically more diverse than that of a simple thioglucosidase action accompanied by Lossen rearrangement with concomitant sulfate displacement and formation of *iso*thiocyanates [6]. The present paper presents hitherto unreported observations which suggest that both the pathway as well as the rate of degradation of these thiohydroximyl sulfates are dependent upon the pH of the medium and upon the presence of foreign protein and ascorbic acid.

A myrosin-sinigrin reaction mixture develops a turbidity at pH 3.0 (glycine-HCl or HCl) (but not at pH 4 or above) which increases with time until a maximum value is reached (Fig. 1). The results

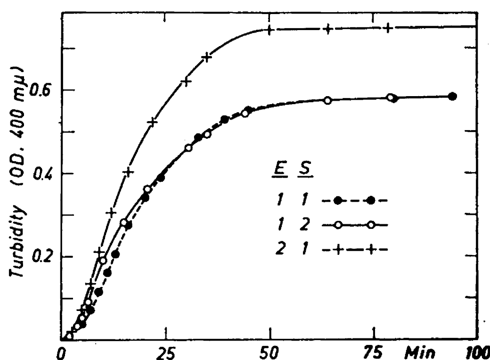

Fig. 1. Development of turbidity in a sinigrin-myrosin reaction mixture at pH 3.0 (0.033 M glycine-HCl). Six ml of reaction mixture at 25° contained 10 μmoles of sinigrin (S = 1) and 1 mg of myrosin (E = 1). Myrosin was prepared by lyophilization of the precipitate obtained from the dialyzed protein fraction (of a water extract of ground black mustard seed) precipitating between 30 and 70 % ethanol. Turbidity measured as optical density at 400 mμ using an Evelyn Colorimeter.

of Fig. 1 show that doubling the enzyme concentration caused an increase in both the rate and the extent of the development of the turbidity, whereas doubling the substrate concentration did not affect these parameters to any appreciable extent. The turbidity did not develop in the absence of substrate at this pH, nor in the presence of glucose, $KHSO_4$ and allyl *iso*thiocyanate added in amounts equivalent to that of the initial substrate. Nor did this turbidity develop when these end-products were added to sinigrin in the absence of enzyme at this pH. Furthermore, when the myrosin was incubated with sinigrin at pH 5.8 (acetate buffer) at 37° for one hour, or at pH 2.1 for 10 min. no turbidity developed after the pH was adjusted to 3.0.

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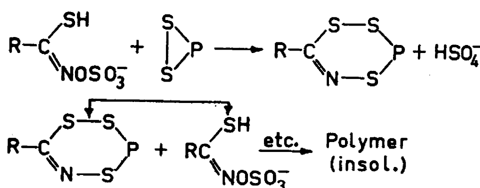
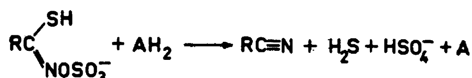
When centrifuged at 100 000 × *g*, a large proportion but not all of the turbid material sedimented. The precipitate gave a positive biuret test (protein) and a negative Molisch test (carbohydrate) whereas the supernatant fraction gave a strong positive Molisch and a much decreased positive biuret reaction. The UV absorption spectrum of the supernatant liquid exhibited no supplementary absorbency at 280 mμ in the background turbidity absorption as one might expect if residual protein had remained in solution.

In corroboration of the observations of Nagashima and Uchiyama [5], ascorbic acid was found to increase the rate of liberation of glucose and the formation of *iso*thiocyanate at pH 5.8 At pH 3.0, in the presence of ascorbic acid in concentrations equivalent to that of the substrate, immediate evolution of hydrogen sulfide (detected by odor and with lead acetate paper) concomitant with the development of turbidity was observed. Exactly the same conditions which were previously observed to produce turbidity also produced hydrogen sulfide in the presence of ascorbic acid. Additional controls in which ascorbic acid was added after extended reaction time at both pH 5.8 and at pH 3.0, did not evolve hydrogen sulfide. A modification of the test for nitriles of Soloway and Lipschitz [7] showed that nitrile was present in an enzyme reaction mixture containing ascorbic acid at pH 3.0 but not at pH 5.8.

Inasmuch as the turbidity appears to rise as the results of insolubilization of the protein of the myrosin preparation during its action and since the existence of a thiol intermediate as a distinct entity at pH 3.0 appears to be likely in view of the above-mentioned findings of Gaines and Goering , it is suggested that at pH 3.0 this intermediate can react with the protein (P) of the myrosin preparation, possibly *via* a chain type sulfhydryl-disulfide interchange as discussed recently by Jansen [8]:



whereas in the presence of ascorbic acid (AH_2), this intermediate decomposes to hydrogen sulfide and nitrile.



Thus the appearance of nitriles in the myrosin-thioglucoside digests, frequently reported in the literature and outlined, *e.g.*, by Kjær [9], may have arisen as the result of the presence of ascorbic acid in unbuffered crude digests. The interpretation of the results herein obtained may reconcile the apparently contradictory conclusions by Nagashima and Uchiyama [5] and those of Gaines and Goering [4]. The former authors concluded that only one enzyme, a thioglucosidase, is present in myrosin preparations, since application of a variety of fractionation procedures and environmental conditions (including low pH-values) yielded a glucose → sulfate ratio of unity. On the other hand, Gaines and Goering [4] have recently presented convincing evidence that myrosin can indeed be separated into two distinct enzymes, an oxime sulfatase and a thioglucosidase and that only the latter acts appreciably on sinigrin at pH 3.0. According to the postulated mechanism of action of myrosin presented herein, a highly purified preparation containing both enzymes but very little foreign protein would liberate and accumulate only glucose and the above mentioned 3-butenyl thiohydroximyl sulfate at pH 3.0 In relatively crude myrosin preparations, this intermediate reacts with the protein impurities as depicted thus liberating inorganic sulfate non-enzymatically.

Detection of nitrile. Both acrylonitrile (the next lower homologue of the postulated end product, vinylacetonitrile) and sinigrin, but not allyl *iso*thiocyanate, reacted positively in the absence of aqueous solvent as described by Soloway and Lipschitz (Procedure II) [7]. Under conditions of the enzyme reaction, in aqueous solution, a brown color developed after boiling for 4 min. in the presence of hydroxylamine, KOH, and propylene glycol when acrylonitrile was present. No color developed in the presence of sinigrin under the same conditions. Upon the subsequent addition of the $FeCl_3$ reagent, the nitrile containing sample developed a muddy brown color. A

sample from the prolonged digestion of sinigrin by myrosin in the presence of ascorbic acid at pH 3.0 gave a positive modified nitrile test, whereas a positive test for neither nitrile nor sinigrin was observed with a sample from a similar enzyme digestion conducted at pH 5.8.

The author wishes to thank Prof. A. Kjær for his encouragement and advice.

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Received June 20, 1960.

Further Observations on the Immunologic Reactions of the Old Yellow Enzyme

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The OYE* and its apoprotein were found to react identically with specific antibodies against the enzyme in agar gel diffusion experiments [1]. The antiserum inhibited the enzymatic activity [2], but no influence was noticed upon the association reaction between the apoprotein and the coenzyme FMN which was studied by fluorimetric technique. The inhibition was non-competitive with regard to the substrate TPNH. These results indicate that the antibodies are bound to other parts of the enzyme molecule than the coenzyme or the substrate.

Atabrine and promazines have been found to inhibit the activity of the OYE after preincubation with the apoprotein [3]. Fluorimetric studies showed that the inhibitors did not prevent the binding of FMN to the apoprotein but slowed down the rate of this reaction. It is suggested that the inhibitors are bound to the apoprotein molecule close to the sites of the FMN. The two different types of inhibitors, drugs and antibodies, thus seemed to exert their effect on different parts of the enzyme molecule. The inhibition studies did not allow any conclusions, however, as to whether the drugs are exclusively bound to sites where they interfere with the coupling of the coenzyme, or whether they affect other parts of the enzyme molecule as well.

In the present work the inhibition of the OYE by anti-serum has been studied at different FMN concentrations. The quantitative precipitation reactions of the OYE and its apoprotein with antibodies, and the influence of atabrine upon these reactions, has been investigated. The effect of the promazines could not be studied, since it was found that precipitation occurred upon mixing promazine or chlorpromazine with rabbit serum.

Materials and methods. The OYE used had been recrystallized once, and the apoprotein was obtained from the same preparation. Immunization with OYE and collection of serum was carried out as described before [1]. The manometric technique used for the inhibition studies has already been described in detail [2]. Quantitative precipitin reactions were performed after the serum had been inactivated at +56°C for 20 min. Varying amounts of antigen in 0.3 ml 0.15 M sodium phosphate buffer pH 7.4 were added to 0.25 ml serum and incubation performed at +37°C for 30 min, followed by 24 h at +4°. After being washed several times in buffer and distilled water, the precipitates were dissolved in 0.3 ml 1 N NaOH and the protein content determined according to Lowry *et al.* [4] The absorption values were compared to those of a bovine serum albumin (Armour) standard. In some experiments antigen solutions containing 0.25 mg OYE or apoprotein per ml were incubated with 2×10^{-4} M atabrine at +37°C for 30 min before being added to the serum. The concentration of atabrine in the antigen-serum mixture was 1.1×10^{-4} M.

* Abbreviations: OYE = old yellow enzyme, FMN = flavin mononucleotide, TPNH = reduced triphosphopyridine nucleotide.