NEW EVIDENCE FOR A TWO ENZYME SYSTEM IN MYROSINASE*

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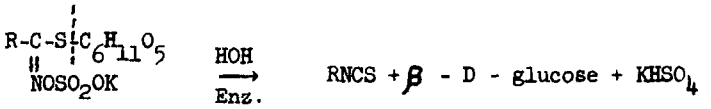
Received February 9, 1960

The uncertainty concerning the activity of myrosinase[1], an enzyme occuring in plants of the mustard family, Cruciferae, has been in question since the discovery of the enzyme in the middle of the last century. The natural substrates for the enzyme are the mustard oil glucosides, of which about thirty are known to occur in nature. The general structure of these glucosides and the products obtained from them by enzymatic cleavage may be represented as follows:



The portion of the aglycone represented by (R) may vary considerably, and appears to reflect only upon the rate of enzymatic hydrolysis. The broken line illustrated above indicates the site of hydrolysis by the thioglucosidase found in the enzyme system.

Early work suggested that the enzyme was composed of two entities, a glucosidase or thioglucosidase and a sulfatase (von Euler and Erikson, 1926). These two enzymes reportedly have been separated and activity obtained from each fraction (Neuberg and Schoenebeck, 1933). Subsequent work has led to the observance of the two enzyme system as the factor involved in the total hydrolysis of these glucosides (Sandberg and Holly, 1932; Ishimoto and Yamashina, 1949).

The elucidation of a new molecular structure for the mustard oil glucosides

*This work was supported by National Science Foundation research grant G-7497.

[1]Throughout the literature the name myrosinase has been used analogously with the names myrosin and sinigrinase.

established some doubt as to the necessity of the two enzyme system for the hydrolysis of these compounds (Ettlinger and Lundeen, 1956). It has been proposed that a single enzyme, a thioglucosidase, is responsible for total hydrolysis of the substrates (Ettlinger and Lundeen, 1957; Reese, Clapp and Mandels, 1958). These authors have indicated that the substrate molecule undergoes a rearrangement of the Lossen type after the glucose unit is removed by enzymatic hydrolysis.

Recent work suggests that there may be a yet undetected factor involved in the hydrolysis of the mustard oil glucosides (Gmelin and Virtanen, 1959). The observation that the myrosinase system found in certain plants closely related to the mustard family hydrolyzes these glucosides with the liberation of thiocyanates rather than isothiocyanates would indicate that at least two factors are involved in the hydrolysis and rearrangement of these molecules.

It is the purpose of this preliminary communication to report experimental evidence favoring the requirement of the two enzyme system for the total hydrolysis of mustard oil glucosides.

Methods and Materials

The crude enzyme was prepared from defatted Oriental yellow mustard seed, Brassica juncea, by ethanol fractionation (Sandberg and Holly, 1932). This enzyme preparation was further purified by ammonium sulfate fractionation, and only the precipitated fraction containing the highest percentage of the total activity was retained for further use. The active fraction, after removal of the salt, was subjected to chromatography on N,N'-diethylaminoethylcellulose (DEAE) and eluted with gradient citrate buffer. The fraction was also checked for purity by boundary electrophoresis in a Perkin-Elmer model 38-A electrophoresis apparatus.

Sinigrin (R equal to $CH_2=CHCH_2-$), which was used for checking the activity of all fractions, was purchased from the California Corporation for Biochemical Research. It was chromatographed on paper in several solvent systems and gave only a single reducing spot.

For analysis of activity, 2.0 mg. of substrate in 0.5 ml. buffer were in-

cubated with 0.2 ml. of enzyme solution. Citrate buffer, pH 6.2 was used for both enzyme and substrate solutions. The reaction vessels were incubated at 37 degrees C. for four hours and the reactions stopped either by heating in boiling water for 5 min. or by the addition of 1.0 ml. of 20% tricloracetic acid. Glucose was determined by the dinitrosalicylic acid method (Sumner, 1925; Methods in Enzymology, 1955) and inorganic sulfate by precipitation as benzidine sulfate (Dodgson and Spencer, 1953). Isothiocyanate was converted to thiourea and determined colorimetrically by the use of Grote's reagent (Grote, 1931).

Results and Discussion

The following results lend conclusive support to the concept of a two enzyme system involved in the total hydrolysis of the mustard oil glucosides.

Ammonium sulfate fractionation of the crude enzyme preparation increased the specific activity for the total hydrolysis (mg. products liberated per mg. protein) by a factor of about four thousand. Chromatography of the salt fractionated enzyme yielded two peaks, as did electrophoresis, Figure 1. The mobilities of the peaks in veronal buffer, pH 8.5, at 0.1 ionic strength were $-.863 \times 10^{-5}$ cm.^{2} volt^{-1} sec.$^{-1}$ (small peak) and 2.588×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$

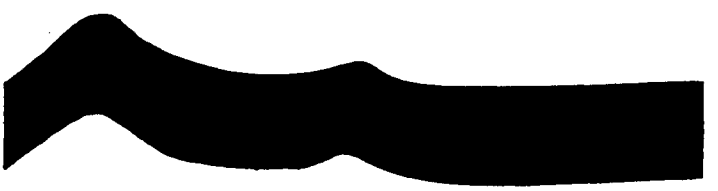


Fig. 1. Electrophoresis pattern of purified myrosinase. Ascending boundary in veronal buffer, pH 8.5, 0.1 ionic strength; time = 1 hour, 49 min. (see text for mobilities)

Further purification, i.e., separation of the components of the system, essentially prevented any total hydrolysis of the substrate. The partial hydrolytic activity attributed to each component liberated approximately the same quantity of inorganic sulfate and glucose respectively as was released during total hydrolysis.

Each fraction from the DEAE column and electrophoresis cell was checked for activity on sinigrin. One fraction, that with the positive mobility, brought about the liberation of glucose without formation of either inorganic sulfate or isothiocyanate in measurable quantities. The negative migrating fraction liberated inorganic sulfate without glucose or isothiocyanate. The combined fractions possessed activity for the total hydrolysis of the substrate. (See Table I).

TABLE I

Hydrolysis of Sinigrin by Separate and Combined Myrosinase Fractions

Enzyme System[a]	Micrograms Glucose	Micrograms Inorg. SO_4	Micrograms Thiourea
Large peak			
(1)	185	0	0
(2)	195	0	<10
Small peak			
(1)	0	75	0
(2)	0	81	0
Combined pks.			
(1)	235	108	159
(2)	250	100	150

See text for assay procedures[b]

a. Large peak designates the positive migrating fraction identified with the thioglucosidase; the small peak is the negative migrating fraction described in the text. (1) and (2) represent duplicate enzyme fractionation.
b. Zero time controls were run on all samples and measured against reagent blanks.

Aliquots of the purified enzyme system were incubated with sinigrin at pH 6.2 and pH 3.0. At pH 6.2 all of the hydrolysis products of sinigrin could be detected. At pH 3.0 the activity of the thioglucosidase was approximately 30% retained, while the activity of the sulfatase was essentially lost, only a negligible amount of inorganic sulfate could be detected. No isothiocyanate

could be detected at the lower pH.

Phosphate brings about considerable inhibition of the thioglucosidase, about 41%, without appreciable effect on the sulfatase. This result appears to explain some of the early work in which the liberation of glucose did not reach the theoretical maximum, whereas the liberation of inorganic sulfate approached complete hydrolysis when the system was incubated in phosphate buffer.

It is also of interest that the sulfatase system has been found to possess hydrolytic activity toward several synthetic substrates, sulfonated oximes, but has no activity on ethereal sulfates or glucose-6-SO_4.

The results of our experimentation indicate definitely that there is a sulfatase, or factor which possesses sulfatase activity, involved in the total hydrolysis of the mustard oil glucosides. The mechanism by which the total hydrolysis takes place is at this time unknown. From the data obtained thus far it appears that after enzymatic removal of the glucose moiety by the thioglucosidase, the residual structure undergoes cleavage of the sulfate group and subsequent rearrangement, probably while bound to the sulfatase factor. Experimentation is underway at present to further elucidate this mechanism. It has been suggested that sulfatases may not be true hydrolytic enzymes, but may actually be sulfate transferases (Dodgson and Spencer, 1956). The undetected factor proposed by Gmelin and Virtanen is not apparently associated with the sulfatase fraction, but may perhaps be a third entity involved in the complex system.

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