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The Preparation of Highly Purified Spinach Leaf Catalase

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The enzyme catalase has now been prepared in crystalline form from many different sources: the liver of beef, horse, man, guinea pig and lamb, the erythrocytes of beef, horse and man, the kidney of horse and the cells of the bacterium *Micrococcus lysodeikticus*. (For references, see Herbert and Pinsent [1] and Bonnichsen [3, 2]). In general, catalase from animal sources is quite stable and convenient to handle, but in sharp contrast with this, the catalase of green plants has been found by numerous investigators to be very labile. Only in the work of Embden [4] has a relatively stable plant catalase extract been reported. However, this investigator used as a source of catalase pumpkin seedling cotyledons, from which it would be difficult to prepare enough material for isolation or for chemical characterization of the enzyme.

As a result of the lack of a readily available source of stable plant catalase, our knowledge of the nature of the enzyme is very scanty. We know only that it rises and falls suddenly several days after germination (Embden [4], Zeile [5], Holman [6]), that chloroplasts contain most of the activity of the leaf (Neish [7]), that the enzyme is inhibited by cyanide and gives a faint hematin spectrum (Zeile [5], Keilin and Hartree [8]), and that it is stable between pH 6.8 and 8.2 (von Euler, Myrbäck and Myrbäck [9]).

The present study was undertaken (a) to attempt to find a convenient source of stable plant catalase, (b) to purify the enzyme as much as possible, (c) to obtain information as to the chemical nature of the enzyme.

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EXPERIMENTAL

A. Estimation of catalase activity

All assays for catalase activity described in this paper were made by the rapid titration method of Bonnichsen, Chance and Theorell [10]. Essentially, this is a modification of the von Euler and Josephson [11] technique, and is based on the titration of H_2O_2 by $KMnO_4$ before the addition of catalase and at frequent intervals after its addition. In our experiments, from 0.01 to 0.2 ml of the extract was assayed, depending on the activity of the particular sample. The aliquots for titration were removed at 15 and 30 seconds from the active preparations, and at 1 and 3 minutes from the less active ones. From the titration data, a monomolecular k value was calculated according to the expression

$$k = \frac{1}{t} \log_{10} \frac{x_0}{x_t}$$

where x_0 is the titer at time zero, x_t the titer at time t and t the time in minutes which the enzyme has acted on the H_2O_2.

The relative purity of each fraction was expressed in terms of the *Kat.f.* of von Euler and Josephson [11], where

$$Kat.f. = \frac{k}{\text{gms. enzyme used in the test}}$$

To obtain the dry weight of the proteins per ml of sample used, 1 ml of the material was dialyzed two times against 2L of distilled water for 24 hours. The sample was then pipetted on to a tared watch glass, dried overnight at 105° C and weighed on an analytical balance.

B. Preparation of the enzyme

Spinach leaves were chosen as a starting material because of the ready availability of large quantities of this product, and because preliminary tests showed the presence of active catalase in crude extracts. 75 kilograms of freshly harvested spinach * were washed in running tap water aad stored in a refrigerated room maintained at + 4° C. About 3 days after harvest, the leaves were transferred to large stainless steel vats containing commercial acetone chilled to — 15° C in a freezing room. After at least two hours of storage under the

* Purchased from T. Wistrand, Lindholmen.

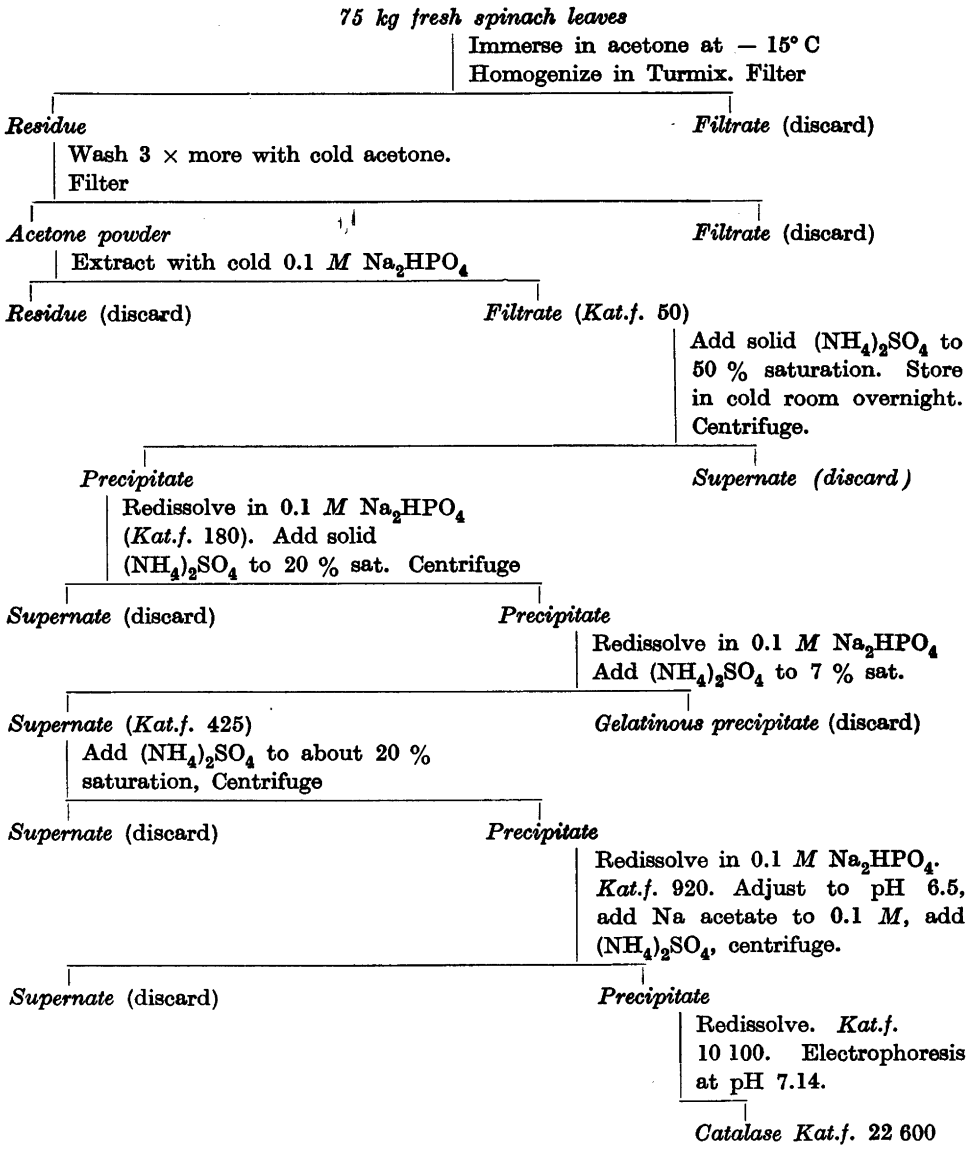


Fig. 1. Scheme for the fractionation of spinach leaves to yield a highly purified catalase.

cold acetone, samples of the leaves were removed, placed in a large Turmix apparatus, covered with some of the cold acetone, and blended vigorously for 30 seconds. Since the leaves were rendered very brittle by the cold acetone,

even the brief blending treatment sufficed to reduce them to a fine slurry. The slurry was decanted into another large stainless steel tub kept in the cold room. After the entire 75 kilograms of spinach had been blended, the slurry was stirred vigorously, filtered through a perforated stainless steel funnel lined with fine muslin, and the green filtrate discarded. The precipitate was once more treated with about 20 liters of cold acetone, again filtered through muslin, and the filtrate again discarded. The residue was then transferred to large glass funnels lined with two layers of filter paper, washed several more times with cold acetone, and allowed to drain for several hours in the cold room. The partially-dried residue was then spread out on large sheets of filter paper in a well-ventilated hood and allowed to dry overnight. The result of this treatment was about 6 kilograms of greenish powder. Occasional grayish lumps in the powder reduced readily to the powder form when rubbed between the hands.

The further fractionation of this acetone powder can be followed by reference to Fig. 1. The powder was first placed in a stainless steel tub, mixed with 10L of cold 0.1 M Na_2HPO_4, and stirred vigorously with a motor stirrer for one hour. The slurry was then filtered by gravity in the cold room through two layers of filter paper. The residue of the filtration was washed at least three more times with additional portions of the cold Na_2HPO_4. The total volume of the filtrate at this point was approximately 25 liters, and had a *Kat.f.* of about 50. The residue of this extraction still contains considerable catalase, which continues to be slowly extracted by large volumes of buffer. For the sake of convenience, however, the extraction was halted when the main bulk of the catalase had been obtained in the extract.

The 25L of extract were then half-saturated with $(NH_4)_2SO_4$ by the addition of the solid salt, and the solution permitted to stand in the cold room overnight. The next morning, a surface layer of lipoidal material was skimmed off and discarded, and the remainder of the solution centrifuged for 30 minutes at 2 000 rpm in an International Serum Centrifuge having a capacity of 13 L. Almost all of the catalase activity was in the precipitate, and the brownish supernatant liquid was therefore discarded. The ammonium sulfate precipitate was then redissolved in 0.1 *M* Na_2HPO_4, allowed to stand overnight in the cold room, and recentrifuged. The supernatant liquid was saved, and the precipitate was again mixed with cold Na_2HPO_4, centrifuged, and the supernatant liquid combined with the first supernatant. The *Kat.f.* at this point was 180 and the total volume about 4L.

To the clear supernatant, saturated $(NH_4)_2SO_4$ was now added dropwise with vigorous stirring. At about 12 % saturation with the ammonium sulfate, a silkiness developed. This was deposited by prolonged centrifugation, and

proved to be colorless rhomboidal crystals devoid of catalase activity. The nature of this material was not further investigated. Ammonium sulfate was again added dropwise, and at about 20 % saturation, a copious precipitate deposited. This was sedimented by centrifugation for 1 hour at 3 000 rpm. in a water-cooled Collatz centrifuge. The supernatant liquid was practically without activity and was discarded.

The precipitate was redissolved in cold 0.1 *M* Na_2HPO_4 and once again subjected to ammonium sulfate fractionation. This time, at about 7.5 % saturation, a copious gelatinous precipitate deposited which was devoid of catalase activity, and was removed by centrifugation. The *Kat.f.* of the resulting clear solution was 425. More ammonium sulfate was then added, and at about 20 % saturation a fine precipitate appeared which contained almost all of the catalase activity. The *Kat.f.* of this material was 920. The material also showed a faint absorption band at about 630 mμ when viewed through a hand spectroscope. Its absorption spectrum, as obtained in a model DU Beckman spectrophotometer, showed strong peaks at 275 and 330 mμ and weaker maxima at 405 and 625 mμ.

This catalase solution was now adjusted to pH 6.5 and made 0.1 *M* with respect to sodium acetate by the addition of the solid salt. Cautious fractionation with saturated ammonium sulfate resulted in the deposition of several precipitates, ranging in *Kat.f.* from 1 200 to 10 100. A total of 19 ml of the most active material was obtained, with a protein concentration of 24.6 mg per ml.

Various techniques were now tried on small aliquots of the enzyme in an attempt to achieve further purification of this material. Some of the techniques failed to purify at all and others gave such poor yields of the purified material as to be impractical as preparative procedures. Among the techniques surveyed were fractionation by cold ethanol, cold acetone, alcohol-chloroform, K_2HPO_4, $(NH_4)_2SO_4$, and $(NH_4)_2SO_4$ plus sodium acetate; also adsorption on charcoal, solid $Ca_3(PO_4)_2$ column, $Ca_3(PO_4)_2$ gel, and several ion-exchange resins. It was therefore decided to subject the material to electrophoretic analysis, with a view to use electrophoresis as a preparative method.

A preliminary run was made with catalase of *Kat.f.* 920 which had been dialyzed for two days against two changes of pH 7.14 phosphate buffer of ionic strength 0.1. Four electrophoretic components appeared, two major colored components and two minor uncolored components. All migrated toward the anode. After 4 hours of electrophoresis (18 milliamperes, 330V), it was possible to obtain samples enriched in each of the colored constituents. These were assayed for catalase activity and were also subjected to spectrophotometric investigation. The faster moving fraction had a *Kat.f.* of 797

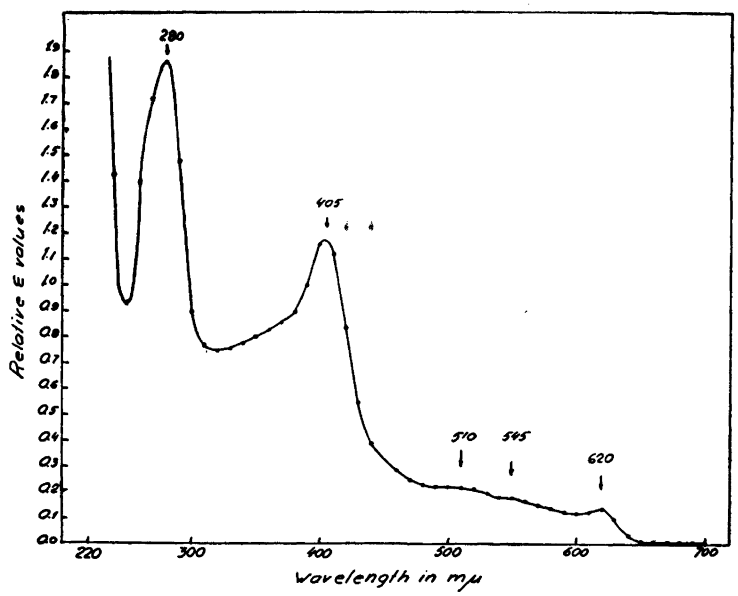
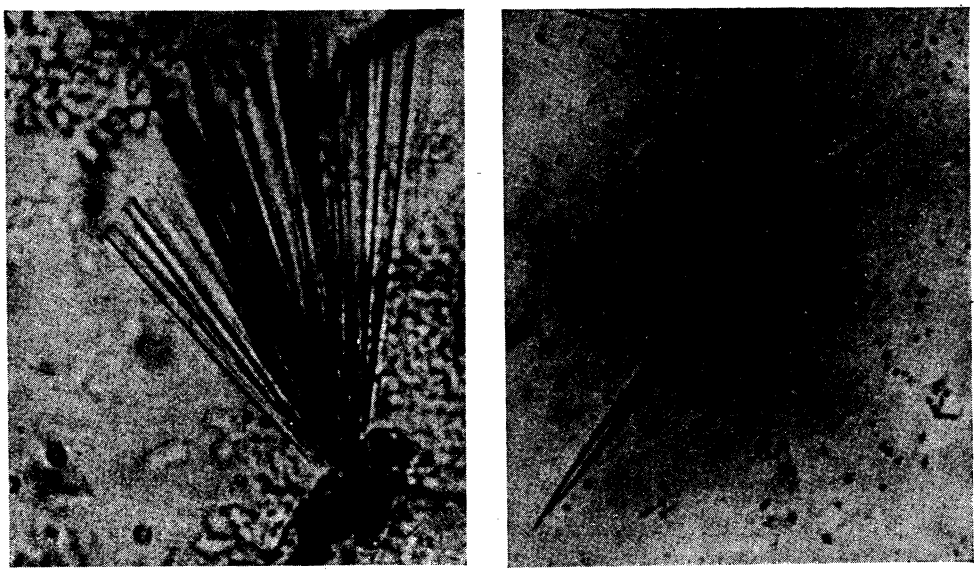


Fig. 2. The absorption spectrum of electrophoretically prepared spinach leaf catalase. Kat. f. = 23 600. Concentration of the enzyme approximately 0.5 mg per 1 ml.

and a ratio $\frac{E_{280}}{E_{405}}$ of 2.61. The slower moving component had a *Kat.f.* of 1 548 and a ratio $\frac{E_{280}}{E_{405}}$ of 1.97. Obviously, the slowermoving component was enriched in catalase.

Another electrophoretic run was then made with the catalase of *Kat.f.* 10 100 which had been dialyzed for 2 days against pH 7.14 phosphate buffer of ionic strength of 0.1. Five fractions were collected, three on the anodic side, one in the bottom cell, and one from the cathodic side. They were each assayed for catalase activity and were also subjected to spectrophotometric analysis in the Beckman spectrophotometer. The results of these analyses are shown in Table 1. It is clear that the cathodic fraction is greatly enriched in catalase. An absorption spectrum of this material is shown in Fig. 2. It shows peaks at 280, 405, 510, 545 and 620 mμ and has a ratio $\frac{E_{280}}{E_{405}}$ of 1.54. This ratio is still considerably higher than that found in blood and liver catalases, in which the 405 mμ peak is actually higher than the 280 mμ peak.

Attempts were now made to crystallize the enzyme. The purified catalase was first concentrated to one-third its volume by placing it in a cellophane



A *B*

Fig. 3. Crystals of the presumptive plant catalase together with the amorphous material which also precipitates (440 x). A = occasional clusters of needles. B = abundant isolated needles or needle fragments.

Table 1. Catalase activity and spectrophotometric characteristics of electrophoretically obtained spinach catalase fractions.

Fraction	Kat.f.	Ratio E_{280}/E_{405} mμ
− 2	23 600	1.54
Bottom cell	9 020	2.07
+ 2	12 100	2.07
+ 1	7 320	2.37
+ (top cell)	2 620	2.54

dialysis bag and blowing air gently over the surface of the bag. The concentrated material was then dialyzed in the cold room against 0.1 *M* Na_2HPO_4 to which small increments of saturated ammonium sulfate were added every few hours. The solution became quite silky at an ammonium sulfate concentration of about 12 %. It was permitted to stand in the cold room for two days, and the fine precipitate sedimented in the Sorvall high-speed centrifuge. The precipitate contained numerous clusters of needle-like crystals (Fig. 3), together with a greater quantity of amorphous material. When the precipitate was redissolved in 0.1 *M* Na_2HPO_4, it gave a good catalase spectrum and had

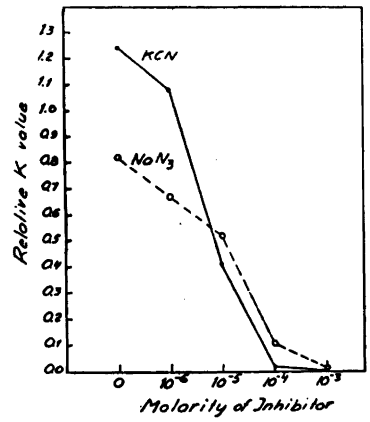


Fig. 4. The effect of KCN and NaN_3 on the activity of spinach leaf catalase.

a *Kat.f.* of 18 600. Other precipitates obtained from the mother liquor had similar or lower *Kat.f.* values.

Although it is highly probable that the crystals portrayed in Fig. 3 are actually crystalline plant catalase, this cannot be stated with certainty, since some amorphous material was present in all the fractions obtained. Thus far, all attempts to separate the crystals from the amorphous matter by differential centrifugation and differential solubilities have been unsuccessful.

C. Experiments with the purified enzyme

Small aliquots of the purified catalase were employed for various studies designed to yield information as to the nature of the enzyme.

1. *Inhibition studies.* Heme-containing enzymes are typically inhibited by cyanide and azide. Our experiments with spinach catalase showed clear inhibition by KCN and NaN_3. As seen from Fig. 4, a KCN concentration of 5×10^{-6} *M* produces a 50 % inhibition of the enzyme, and 10^{-4} *M* produces essentially complete inhibition. NaN_3 is somewhat less effective, half inhibition being produced by 2×10^{-5} *M* and complete inhibition by 2×10^{-4} *M*. Na diethyldithiocarbamate, a typical copper-enzyme inhibitor, is without effect on the enzyme.

2. *Identification of the prosthetic group.* The prosthetic group of heme containing enzymes can in most cases be removed by dilute HCl in acetone. After such a splitting, recombination experiments and identification of the prosthetic group can be attempted. 1 ml of the spinach enzyme was treated with 3 ml of cold 0.1 % HCl in acetone. The white protein was centrifuged down and redissolved in 1 % $NaHCO_3$. It proved to be devoid of catalase

activity, and could not be reconstituted by the addition of the prosthetic group or of hematin.

The acetone was then removed from the supernatant liquid by blowing a stream of air over it. This resulted in the deposition of a red-brown precipitate, leaving behind a colorless aqueous layer. The precipitate was dissolved in pyridine. To 1.4 ml of the pyridine solution were added 0.3 ml of 1N NaOH and 2.3 ml of H_2O. The mixture was then placed in a Beckman cuvette, and after the addition of crystals of $Na_2S_2O_4$, a sharp absorption peak at 557 mμ appeared. This indicated that the prosthetic group of the plant catalase is protohemin. The fact that the aqueous layer was colorless indicates the absence of bile pigments from the catalase preparation.

3. *Iron assay.* The iron content of the enzyme was determined by the sulfosalicylic acid colorimetric method. To 1 ml of enzyme solution were added 1 ml of concentrated H_2SO_4 and 3 drops of concentrated H_2O_2. The material was digested over a flame, being cooled occasionally to permit the addition of more H_2O_2. After digestion was complete, the solution was cooled and 5 ml of distilled water added. The preparation was then concentrated to 1/3 volume by boiling, decanted into a 10 ml volumetric flask and 0.2 ml of 20 % sulfosalicylic acid added. Concentrated NH_4OH was then added dropwise until the solution became yellow. It was then made up to volume and the absorption at 424 mμ measured in a Beckman spectrophotometer. The sample assayed 0.049 % iron, which is roughly half the value obtained for a pure 4-hematin catalase.

4. *Stability of the enzyme.* The enzyme is completely destroyed by being heated at 60° C for 10 minutes. It is stable in the cold between pH 5.3 and 8.9, being destroyed rapidly at lower pH values and slowly at higher pH values. The activity of the enzyme over a 30 second period is optimal between pH 5.3 and 8.0, falling off rapidly at more alkaline and acid pH values.

DISCUSSION

The experiments described in this paper show clearly that stable plant catalase can be conveniently prepared and considerably purified by conventional techniques. The unambiguous crystallization of this enzyme should now be only a matter of time. The *Kat.f.* of 23 600 obtained for highly-purified spinach catalase is roughly one-fourth that obtained for bacterial catalase and highly-active animal catalase. Preliminary kinetic data obtained by Dr. B. Chance on a very impure sample of our material also indicate a k' (Bonnichsen, Chance and Theorell [8]) roughly 20 % that of other catalases. These facts, coupled with the low iron content (0.049 %) and low ratio of absorpt-

ion at 405 mμ compared with 280 mμ suggests that plant catalase may have less than 4 hematin groups per molecule.

Considerable physiological significance may possibly be attributed to plant catalase in view of the fact that it is apparently localized in the chloroplasts (Neish [5]). Since it has been suggested that the oxygen-releasing system of photosynthesis is a heavy metal enzyme, and since catalase contains a heavy metal and releases oxygen, it may conceivably be involved in the photosynthetic release of oxygen. The possible role of catalase in the mechanism of photosynthesis was recently re-evaluated by Tamiya [12]. Since, also, the catalase content of various plant tissues is known to shift rapidly at the inception of growth and in response to illumination, catalase may somehow be importantly involved in the control of plant growth and form.

SUMMARY

The catalase of spinach leaves has been prepared in a stable, highly purified form. The method of preparation involves reducing the leaves to an acetone powder, extracting with 0.1 *M* secondary phosphate, several ammonium sulfate fractionations, and finally preparative electrophoresis. The maximum *Kat.f.* obtained was 23 600. The spectrum shows peaks at 280, 405, 510, 545 and 620 mμ. The ratio E_{280}/E_{405} is 1.54. The preparation has 0.049 % iron and has a protohemin type of prosthetic group. The enzyme is stable between pH 5.3 and 8.9 and is rapidly destroyed at temperatures above 60° C.

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