ACTA CHEMICA SCANDINAVICA 5 (1951) 815–819

A Highly Active Horse Erythrocyte Catalase

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Purified erythrocyte catalase preparations from various laboratories generally give Kat.F. values of from 50 000—65 000 [1–3]. Agner [4], however, has succeeded in isolating a sample from human red cells by a method utilizing electrophoretic separation in the final purification step which gave a value of 100 000. The human erythrocyte catalase of Herbert and Pinsent [3] gave a Kat.F. of only 60 000. This discrepancy in activity suggests that catalase preparations may be composed of molecules of varying activities and that under certain conditions it should be possible to prepare material having higher activity than the usual Kat.F. 60 000. This possibility has been realized in working with horse erythrocyte catalase and a preparation showing a Kat.F. approaching 120 000 has been obtained. Some of the properties of such material are presented here.

EXPERIMENTAL

Crude horse erythrocyte catalase was prepared by the method of Bonnichsen [2] with some modification of procedure. Initially, sufficient of the ethanol-chloroform mixture was added (from 1 to 1.3 volumes) to washed red cells, previously diluted with 2 volumes of distilled water, to effect a rapid removal of the hemoglobin as in the procedure of Herbert and Pinsent [3]. The final ethanol fractionation step prior to crystallization was omitted since all of the catalase was found to precipitate at the step designed to remove impurities. A series of crystalline fractions were prepared by slowly increasing the $(NH_4)_2SO_4$ concentration. The initial fractions appeared to be contaminated with varying amounts of amorphous material. The more soluble fractions were composed only of crystalline material and were readily recrystallized. If dilute solutions were slowly recrystallized from $(NH_4)_2SO_4$ some of these fractions often formed relatively large crystals which dissolved only very slowly upon suspension in distilled water. A microphotograph of such a suspension is shown in Fig. 1. These crystals resemble those obtained upon storage of relatively concentrated aqueous catalase solutions in the cold [2,5].

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Two attempts to utilize the $Ca_3(PO_4)_2$ adsorption method of Herbert and Pinsent [3] for the preparation of horse erythrocyte catalase were unsuccessful. This method was, however, found to be applicable to human erythrocytes as described by these workers.

The absorption spectrum of the catalase preparations was determined in a Beckman spectrophotometer using $M/150$, pH 6.8 phosphate as solvent. Hemin was determined as alkaline-pyridine-hemochromogen and iron as ferric sulfosalicylate in ammoniacial solution.

Kat.F. determinations were carried out by both the method of von Euler and Josephson [6] and of Bonnichsen, Chance and Theorell [7]. These methods gave very comparable results, the values usually agreeing within 5 %. Because of its ease and rapidity of performance most activity measurements were carried out as recommended by the latter workers.

EXPERIMENTAL RESULTS

A single preparation of catalase was utilized in these studies. It represented the final crystalline fraction obtained from 14 liters of washed horse erythrocytes and amounted to 12 ml of an 0.89 % solution. This amount represents approximately 5 % of the usual yield.

Activity: When first tested this catalase preparation gave an average Kat.F. of 117 000. It gave a first order reaction constant at 18° C of 6.64×10^7 liter $\times$ mole $^{-1} \times$ sec^{-1} when assayed by the rapid method of Bonnichsen, Chance and Theorell [7]. This may be compared to these workers' value of 3.5×10^7 at 22° C for horse erythrocyte catalase of Kat.F. 61 000. Consequently our catalase showed a Kat.F. of 116 000 by this method if the temperature coefficient of this reaction, which is low [7], is neglected. The average value obtained by the method of von Euler and Josephson [6] was 118 000.

Enzyme lability: After standing for 18 days in the refrigerator the preparation gave a first order reaction constant of 4.88×10^7 mole $\times$ liter$^{-1} \times$ sec $^{-1}$. This decrease in activity of 27 % is an example of the well-known lability of catalase in dilute solution. Another example of this type of lability is seen upon dilution of the solution for assay. A typical result of the assay of the 0.89 % catalase at various times after ten-fold dilution is as follows:

Time after dilution (min.)	K_1 *
Initial	1.29×10^{-2}
5	0.87×10^{-2}
15	0.88×10^{-2}

* Given by 0.1 ml of a 1—10 dilution of the above 0.089 % solution under the usual conditions of the test [7].

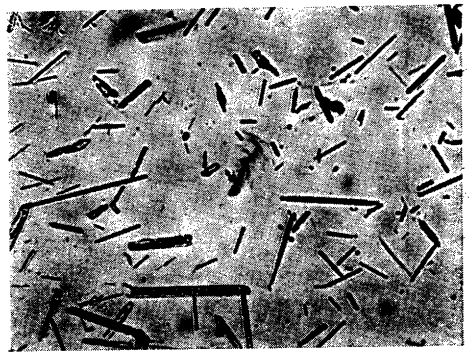

Fig. 1. Photomicrograph of catalase crystals from ammonium sulfate (magnification 130 ×).

Approximately 30 % of the activity was lost between the initial and 5 minute assay. The activity then remains quite constant. This suggests that only a portion of the catalase is very labile or that the denaturation reaction that takes place rapidly attains an equilibrium. As the activity of the 0.89 % solution decreased with age it appeared to be less sensitive to this dilution effect. Dilution with glass distilled water or bovine serum albumin solution did not abolish or retard this destruction.

Since the initial activity determination cannot be conducted in less than 30—60 seconds after dilution, it is likely that the first reaction rate constant is also low. Hence the original Kat.F. value of this preparation must be regarded as minimal. A truer picture of the activity of such potent but labile catalase preparations will require a rapid assay which circumvents the high dilution step.

Even in the rapid method of assay, the enzyme activity is seen to fall off slightly with time, a decrease in activity of 5—10 % in 45 seconds usually resulting. An example of this is shown by the data of Fig. 2. For this reason the results of the 15, 30 and 45 second titrations were extrapolated to zero time in activity determinations.

Absorption studies: This catalase gave a molar extinction coefficient (β) of 89.4 and 68.8 $\times$ 10^7 $cm^2 \cdot mole^{-1}$ at 405 and 277 mμ respectively. This gives a value of 1.3 for the ratio of the 405 to 277 mμ extinctions. The absorption coefficients are slightly higher than those reported by Bonnichsen [8], particularly at 405 mμ. The above ratio for this workers preparation was approximately 1.26. Increases in this ratio should be synonomous with increased catalase purity. We have obtained two other horse erythrocyte catalase preparations giving a ratio for the above extinctions of 1.31. These fractions were obtained in larger yield than the presently discussed material and gave Kat.F. values of only 84 000 and 94 000. Apparently very pure catalases as judged by absorption data can be obtained which show variable activity.

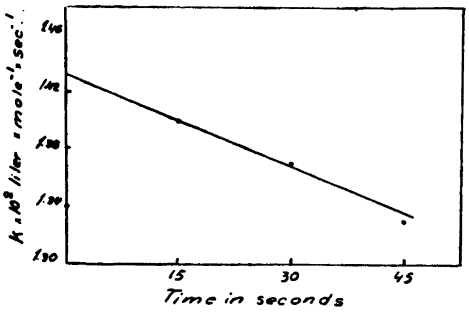

Fig. 2. The decrease in activity of catalase during determination by the rapid method (7).

Hemin and iron content: A hemin content of 1.04 % and an iron level of 0.097 % were found. The hemin value is slightly lower than the values reported by Bonnichsen [8], for horse erythrocyte catalase of Kat.F. 61 000—65 000. This is somewhat surprising in view of the strong absorption at 405 mμ. Other preparations showing ratios of 1.30—1.31 for the extinctions at 405 and 277 mμ have given essentially the same hemin values.

Other studies: A velocity sedimentation and diffusion experiment were performed by Dr. K. O. Pedersen of Uppsala University. The protein showed a single boundry in the ultracentrifuge and gave values for both the sedimentation and diffusion constants which were close to those reported for other catalases [9–11]. More detailed molecular kinetic investigations of horse erythrocyte catalase are being carried out at present.

DISCUSSION

The data presented here suggest that crystalline erythrocyte catalase preparations are composed of molecules of varying activities. A crystalline fraction of low yield was found to give a Kat.F. close to 120 000. Other and somewhat larger yield fractions also representing the more soluble portions of the usual catalase preparations have given activities as high as 97 000. The human erythrocyte catalase of Agner [4] and the bacterial catalase of Herbert and Pinsent [12] are other examples of Kat.F. 100 000 catalases. Brown [13] has also shown by solubility techniques that beef liver catalase contains material of Kat.F. as high as 180 000. Such results may explain why various investigators have obtained catalases showing different activities. It would appear that the catalase preparations studied to date cannot be considered as representative of pure, native catalase, at least from an activity standpoint.

Two reasons can be readily suggested for the variation in activity of different catalase fractions. It is possible that in nature there exist a series of

catalase molecules with widely divergent activities but with close physical and solubility properties. A second alternative is that the rigorous conditions usually employed in preparing catalase may lead to changes in some of the molecules which results in decreased activity. These changes are likely synonomous with solubility changes which allow for the separation of fractions of varying activity. It would appear that the answers to these possibilities would exist in the preparation of catalase by much milder fractionation methods, particularly one that circumvented the Tsuchihashi [14] conditions for the initial removal of hemoglobin by denaturation with an alcohol chloroform mixture.

The high activity catalase studied here was extremely sensitive to destruction on dilution. This destruction is of such a nature as to suggest that only some of the molecules are extremely labile. Present investigations on the denaturation kinetics of catalase have also revealed that this material as well as other crystalline horse erythrocyte catalase preparations are composed of molecules of varying reactivities. The above lability of this catalase preparation along with the known sensitivity to H_2O_2 and the possibility that present fractionation methods tend to give modified catalases suggests that erythrocyte catalase should not necessarily be considered a "rugged enzyme".

SUMMARY

A catalase preparation with a Kat.F. of at least 117 000 has been studied. It gives a first order reaction velocity constant at 18° C of 6.64×10^7 liter $\times$ mole^{-1} $\times$ sec^{-1}. This material has most of the physical properties of other less active erythrocyte catalase preparations. It shows an initial rapid rate of destruction upon dilution in buffers for assay purposes.

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Received February 8, 1951.