Crystallization of glucose oxidase from *Penicillium amagasakiense*

Several investigators[1-3] have reported methods of obtaining highly purified preparation of glucose oxidase (notatin) from *Penicillium* cultures and some of the properties of their preparations. A crystalline preparation, however, has not yet been described. Recently, some of the present authors succeeded in obtaining a new strain of Penicillium, *Penicillium amagasakiense*, and found that this strain excreted glucose oxidase into the culture medium in submerged cultures. The culture conditions for the production of glucose oxidase by this *Penicillium* and the microbiological nature of this strain will be published elsewhere. A crude preparation of this enzyme thus obtained is now commercially available from Nagase and Co., Ltd., under the name of "Deoxine". In the present communication the purification of crystalline glucose oxidase from "Deoxine" and some of its properties are described.

100 g "Deoxine" was dissolved in 100 ml 0.1 *M* sodium acetate buffer, pH 4.5, and dialysed against the same buffer overnight at 5°. Insoluble materials were removed by centrifugation and the clear dark-brown supernatant fluid was passed through a column (5 × 25 cm) of Amberlite CG-50, Type II, which had previously been buffered at pH 4.5 with the same buffer. The enzyme and coloured materials were completely adsorbed on the resin. The column was washed with 2 or 3 l of the same buffer until the eluate was colourless. The first part of the washings contained half the total oxidase activity and was clear yellow. It seems likely, however, that this fraction contains a partially modified enzyme, since on further purification of this fraction it is difficult to obtain a crystalline preparation. The native fraction could be eluted with 0.1 *M* sodium acetate buffer, pH 5.0, from a clear yellow band. Other coloured impurities were scarcely eluted by this treatment. To the yellow fraction, ammonium sulfate was added and the fraction precipitating between 60 and 80 % saturation was collected by centrifugation. The precipitate was dissolved in a minimum amount of 0.05 *M* sodium acetate buffer, pH 5.0, and dialyzed overnight against the same buffer at 5°. The dialyzed solution was passed through a column (2 × 70 cm) of Amberlite CG-50, Type II, which had previously been buffered at pH 5.0 with the same buffer, and the enzyme adsorbed on the column was eluted with the same buffer. The main fraction was collected and fractionated with ammonium sulfate as described the above. The precipitate was carefully dissolved in a minimum amount of water and centrifuged. To the deep-yellow coloured supernatant ammonium sulfate was slowly added until a slight turbidity appeared after the salt had been dissolved. The mixture was left at room temperature and crystals began to appear within a day. Crystallization was complete within 2 or 6 days. The crystals appeared as needles as shown in Fig. 1, and were collected by centrifugation and dissolved in a minimum amount of water (272 mg of crystals, yield 13 %). Recrystallization was carried out as described above.

As shown in Fig. 2, the absorption spectrum of crystalline glucose oxidase showed maxima at 278 mμ, 380 mμ, and 460 mμ in the oxidized form and at 273 mμ in the presence of glucose. The peaks at 380 mμ and 460 mμ due to FAD disappeared on reduction of the enzyme with glucose or $Na_2S_2O_4$.

The preparation was completely homogeneous by ultracentrifugal analysis and did not contain catalase.

Abbreviations used: FAD, flavin-adenine dinucleotide; FMN, flavin mononucleotide.

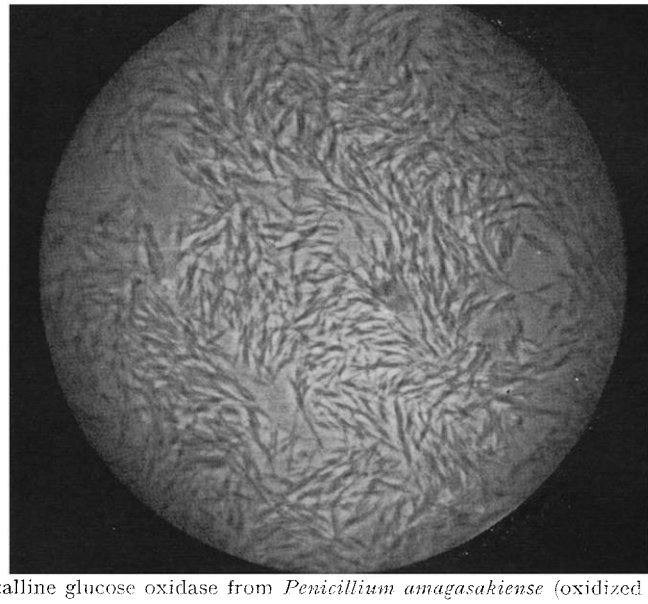

Fig. 1. Crystalline glucose oxidase from *Penicillium amagasakiense* (oxidized form; × 400).

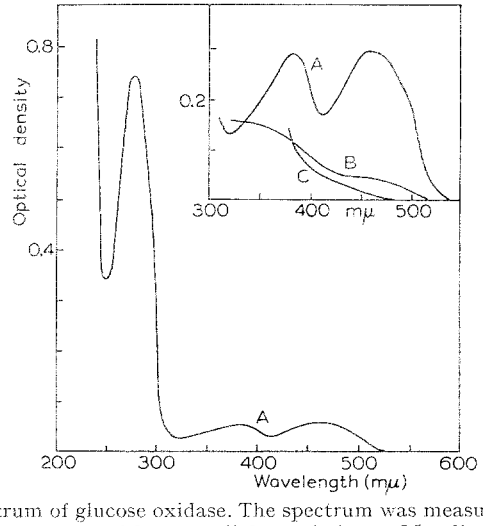

Fig. 2. Absorption spectrum of glucose oxidase. The spectrum was measured with a Cary spectrophotometer Model 14 in a cuvette with a 1-cm light-path, in 0.1 *M* sodium acetate buffer, pH 5.6. Curve A, oxidized form; Curve B, reduced with glucose; Curve C, reduced with $Na_2S_2O_4$.

The sedimentation coefficient ($S_{20,w}$) was 7.93 S and the diffusion coefficient ($D_{20,w}$) was 5.02 × 10^{-7} cm^2/sec based upon determination at four different concentrations of enzyme (0.8–0.2 %) in 0.1 *M* sodium acetate buffer, pH 5.6. The partial specific volume was 0.75. From the above results, the molecular weight was calculated as 154,000. The amount of FAD was found to be 12.8 mμmoles/mg protein by means of the D-amino acid oxidase apo-enzyme test, and the absorbancy at 460 mμ. This result indicates that the equivalent weight per mole of FAD was 78,000. Thus, the enzyme contains 2 moles FAD/mole of enzyme protein. These results are in good agreement of those of CECIL AND OGSTON[4]. The enzyme was stable between pH 3.5

and 7 at 40° and the pH optimum for the glucose-oxidizing activity was at 5.6 at 30°. The activity was measured manometrically in a Warburg apparatus at 30°. The reaction mixture contained 0.2 ml of enzyme solution, 1.2 ml 0.1 *M* sodium acetate buffer, pH 5.6, and 0.2 ml of catalase solution (prepared by the method of KEILIN AND HARTREE[2]) in the main compartment, 0.2 ml 20 % glucose in the side arm, and 0.2 ml 20 % KOH in the center well. Under these conditions, the turnover number (moles O_2 consumed/mole enzyme/min) was 17,000 (the Qo_2 (μl O_2 uptake/mg protein/h) = 148,364 at 30°). The enzyme solution at pH 5.6 could be stored for 2 weeks at 5° without any loss of the activity. The flavin-adenine dinucleotide could be removed from the protein at below pH 2.8 at 0° in the presence of 80 % satd. ammonium sulfate. The activity was regained by the addition of FAD, in proportion to the amount of added FAD. Reactivation was complete on addition of an equal amount of FAD to that liberated. FMN had no effect on the reactivation. The activity was completely inhibited by 10^{-3} *M* *p*-chloromercuribenzoate and partially by 10^{-3} *M* aldehyde reagent such as dimedon, phenylhydrazine, N_2H_4, $NaHSO_3$ and NH_2OH.

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KIYOSHI KUSAI
ICHIRO SEKUZU
BUNJI HAGIHARA
KAZUO OKUNUKI
SABURO YAMAUCHI
MASASHI NAKAI

Dept. of Biology, Faculty of Science, University of Osaka, Osaka (Japan) and Nagase and Co., Ltd., Amagasaki Factory, Amagasaki (Japan)

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The formation of γ-hydroxy-γ-methylglutamic acid from a common impurity in pyruvic acid

Incubation of a recently purchased preparation of sodium [2-^{14}C]pyruvate with cell-free extracts of *Clostridium propionicum* resulted in the formation of a radioactive dicarboxylic amino acid. Several milligrammes of this product were isolated and identified as γ-hydroxy-γ-methylglutamic acid.

Methods for the cultivation of *C. propionicum* are described elsewhere[1]. Cell-free extracts were prepared by crushing the cells in a Hughes press[2], followed by addition of 2–3 vol. 0.05 *M* potassium phosphate buffer, pH 7.4, and centrifugation at 20,000 × *g* in order to remove whole cells and cell-debris.

The amino acid produced on incubation of [2-^{14}C]pyruvate with the extract was isolated by the following procedure. The incubation mixture was treated with 0.1 vol. 10 % $HClO_4$ and centrifuged to remove protein. The supernatant solution was then