tion, LPO 1, and a minor zone with lower mobility towards the cathode. By subjecting enzyme, treated in this way, to electrophoresis together with isolated LPO 2, the second minor component was shown to have a mobility indistinguishable from LPO 2. No heterogeneity was demonstrated after dialysis against the potassium acetate buffer only. Dialysis of homogeneous LPO 2 against ammonium sulfate, pH 9.4, also resulted in a heterogeneity, the minor component corresponding to LPO 3.

These results strongly indicate that LPO 1 and LPO 2 can, under certain conditions be converted to LPO 2 and LPO 3, respectively. The convertibility of LPO 3 and LPO 4 have not yet been investigated. However, before the identity is finally established between the conversion products and LPO 2 and LPO 3 further characterization of the conversion products is necessary.

Thus it has been shown that a heterogeneous LPO, corresponding to LPO 1, LPO 2, and LPO 3, can be produced from a homogeneous enzyme. It is not yet known, however, if conversion is effected during the purification of the enzyme. But even so, it is still quite possible that the peroxidase is heterogeneous in native cow's milk and that there is some process *in vivo* that corresponds to the effect of the alkaline ammonium sulfate. It is known that cytochrome c from beef heart is heterogeneous in fresh muscle extract [7] and it has been shown in this laboratory that this protein is split into subfractions at condition remarkably similar to those effective for LPO.[8] The cytochrome c subfractions appear to differ, like the subfractions of insulin and corticotropin, only in the number of amide groups.[8-10] There are reasons to believe that a similar explanation can also account for the heterogeneity of LPO, as hydrolysis of amide groups under relatively mild conditions should not be an isolated phenomenon for corticotropin [10,11] and cytochrome c.[8]

There are, however, other possible explanations that must be investigated and work is continuing in this laboratory to further study the heterogeneity and convertibility of LPO.

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1. Carlström, A. *Acta Chem. Scand.* **19** (1965) 2387.
2. Carlström, A. and Vesterberg, O. *In preparation.*
3. Carlström, A. *In preparation.*
4. Morrison, M. and Hultquist, D. E. *J. Biol. Chem.* **238** (1963) 2847.
5. Ornstein, L. and Davis, B. J. *Disc Electrophoresis.* Preprinted by Distillation Product Industries, Eastman Kodak Co., 1962.
6. Broom, J. *Nature* **199** (1963) 179.
7. Flatmark, T. *Acta Chem. Scand.* **18** (1964) 1656.
8. Flatmark, T. *Acta Chem. Scand. In press.*
9. Harfenist, E. J. *J. Am. Chem. Soc.* **75** (1953) 5528.
10. Shepherd, R. G. *et al. J. Am. Chem. Soc.* **78** (1956) 5051.
11. Li, C.H., Geschwind, I. I., Dixon, J. S., Levy, A. L. and Harris, J. I. *J. Biol. Chem.* **213** (1955) 171.

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Crystalline Cytochrome c Peroxidase

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Altschul *et al.*[1] were the first to demonstrate the presence of cytochrome *c* peroxidase (cytochrome *c* H_2O_2:oxidoreductase, EC 1.7.11.1) in baker's yeast, assigning a method for its partial purification. Later, Abrams *et al.*[2] improved the purification procedure, which gave a highly pure preparation. However, the enzyme was obtained in a poor yield. By introducing ion exchange chromatography on cellulose-ion-exchangers as a step in the purification procedure, the yield of the enzyme was improved [3,4] and the method of purification simplified. In this communication a procedure for crystallization of cytochrome *c* peroxidase (CcP) is reported, as well as some preliminary observations on its properties.

Dried baker's yeast was ground and allowed to autolyze mainly as described by Altschul *et al.*[1] The turbid brown solution obtained after centrifugation was first

treated with basic lead acetate, the amount of which was decided by tests with small samples. If too much was added, some of the CcP was lost. With our material around 120 ml of a 19 % solution of $PbAc_2Pb(OH)_2$ was usually required per litre of extract. This procedure increased the stability of the crude enzyme extract by eliminating proteolytic activity to a large extent and leaving the peroxidative activity intact in solution. The pH of the solution was adjusted to 4.2 in order to decrease the proteolytic activity still remaining in the solution during the concentration of the extract, which was performed at room temperature in a rotary evaporator by reducing the volume of the solution to half the original. The enzyme was precipitated from this concentrate with between 30—50 % of acetone in the cold (−5°C). The precipitate was redissolved in cold distilled water, insoluble material removed by centrifugation and the enzyme reprecipitated from the clear brown solution at 35 % of acetone in the cold (−5°C). The precipitate was dissolved in 0.05 M acetic acid buffer of pH 5.0 and put on a DEAE-cellulose ion-exchange column equilibrated with the same buffer in the cold. Inactive material was first eluted with the equilibration buffer, after which the enzyme was removed by gradient elution arranged by allowing a 0.3 M acetate buffer of pH 5.0 to flow into a mixing chamber containing the starting buffer. The enzyme preparation was concentrated after dialysis on a short (2 cm) DEAE-ion-exchange column and eluted with 0.3 M acetic acid buffer (pH 5.0). When a concentrated solution of the enzyme was dialyzed against distilled water in the cold, a cloudiness was observed after some time and after two days in the cold a homogeneous mass of large brown crystals could be observed (Fig. 1).

Fig. 1. Crystalline cytochrome *c* peroxidase, 240 ×.

The enzyme preparation was found to be homogeneous in the ultracentrifuge and to have a concentration-dependent sedimentation constant, the extrapolated value of which was equal to 3.55 S. The diffusion constant was found to be equal to 9.4 F. Electrophoretic analysis in the Tiselius apparatus showed the presence of only one component with an I.P. of 5.0. The orcine test showed that the preparation did not contain carbohydrates. The iron content was found to be about 0.15 % of iron per unit of dry weight, which corresponds to a minimum molecular weight of 37 000. On the basis of the sedimentation and diffusion constants and a value for the partial specific volume of 0.738, the molecular weight was calculated to be 35 000.

The enzyme was found to be stable between pH 3.5 and 9.0. The amino acid content differs slightly from that of other peroxidases, there being a rather high content of tyrosine and tryptophane. This is evidently the reason for the low value of the ratio E_{407}/E_{280}, which was found to be about 1.28.

The prosthetic group of CcP was found to be a protohemin and was more tightly bound to the protein, than is the hemin of horseradish peroxidase, as revealed by splitting experiments with acid acetone.

Detailed reports of these findings will be published in this journal.

1. Altschul, A. M., Abrams, R. and Hogness, T. R. *J. Biol. Chem.* **136** (1940) 777.
2. Abrams, R., Altschul, A. M. and Hogness, T. R. *J. Biol. Chem.* **142** (1942) 303.
3. Ellfolk, N. *Eleventh Scandinavian Chemist Congress, Turku 1962*, p. 282.
4. Yonetani, T. and Ray, G. S. *J. Biol. Chem.* **240** (1965) 4503.

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