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An effective chromatographic separation of chicken red blood cell coproporphyrinogen oxidase and uroporphyrinogen decarboxylase, two enzymes in heme biosynthesis

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Abstract—Of the heme biosynthetic pathway enzymes, coproporphyrinogen oxidase is one of the least understood. Substrate recognition studies [*Prepr. Biochem. Biotech.* **1997**, *27*, 47, *J. Org. Chem.* **1999**, *64*, 464] have been done using chicken blood hemolysates (CBH) as the source of this enzyme. However, the enzyme uroporphyrinogen decarboxylase is also present in these preparations and separation of these two enzymes from CBH had not yet been achieved. Thus, a substrate ligand column was developed by covalently linking coproporphyrin-III to a sepharose resin following a similar procedure previously used for the purification of uroporphyrinogen decarboxylase [*Int. J. Biochem.* **1992**, *24*, 105]. The ligand–resin chromatography step rapidly separates coproporphyrinogen oxidase from uroporphyrinogen decarboxylase as well as the majority of the hemoglobin.

1. Introduction

Heme and other porphyrins are critical molecules in many biological processes such as oxygen transport and electron transfer reactions. A defect in one of the enzymes in the heme biosynthetic pathway can cause the upstream intermediates to accumulate. Porphyrias are a group of clinical disorders resulting from the accumulation of the intermediates.[4] Porphyric patients often excrete the pathway intermediates through urine and feces. Some of the common symptoms include dark colored urine and photosensitivity, that is, a sensitivity to light.[5] Interruptions in the heme biosynthetic pathway also reduce the amount of heme produced leading to anemia. For example, defects in the gene for coproporphyrinogen oxidase can lead to the manifestation of symptoms of acute porphyria. This is due to a decrease in enzymatic activity and the biological problems associated with this genetic defect are known as Hereditary Coproporphyria (HC). HC occurs in 1/10,000 of Europeans and North Africans and 1/400 of white South Americans.[6] Understanding coproporphyrinogen oxidase at the mechanistic level may lead to enhanced clinical treatment for this disease.

Uroporphyrinogen decarboxylase (URO-D; EC 4.1.1.37) and coproporphyrinogen oxidase (copro'gen oxidase; EC 1.3.3.3) are the fifth and sixth enzymes of the heme biosynthetic pathway, respectively. Uroporphyrinogen decarboxylase is a cytosolic enzyme that catalyzes the decarboxylation of all four acetate side chains on uroporphyrinogen-III to form methyl groups while releasing CO_2 (Fig. 1c). The resulting molecule is coproporphyrinogen-III (copro'gen-III). Coproporphyrinogen oxidase is found in the intermembrane space of the mitochondria and sequentially converts two propionate groups to vinyl groups by oxidative decarboxylation forming the protoporphyrinogen-IX molecule (proto'gen-IX) as shown in Figure 1a. This reaction gives off two CO_2 molecules; however, the mechanism for this reaction is not well understood. Support for this sequential oxidative decarboxylation pathway has come from many sources. The monovinyl intermediate, harderoporphyrinogen, has been detected in bile,[7] bone marrow,[8] and fecal extracts[9] as well as from the rat harderian gland.[10]

Keywords: Uroporphyrinogen decarboxylase; Coproporphyrinogen oxidase; Hemoglobin.
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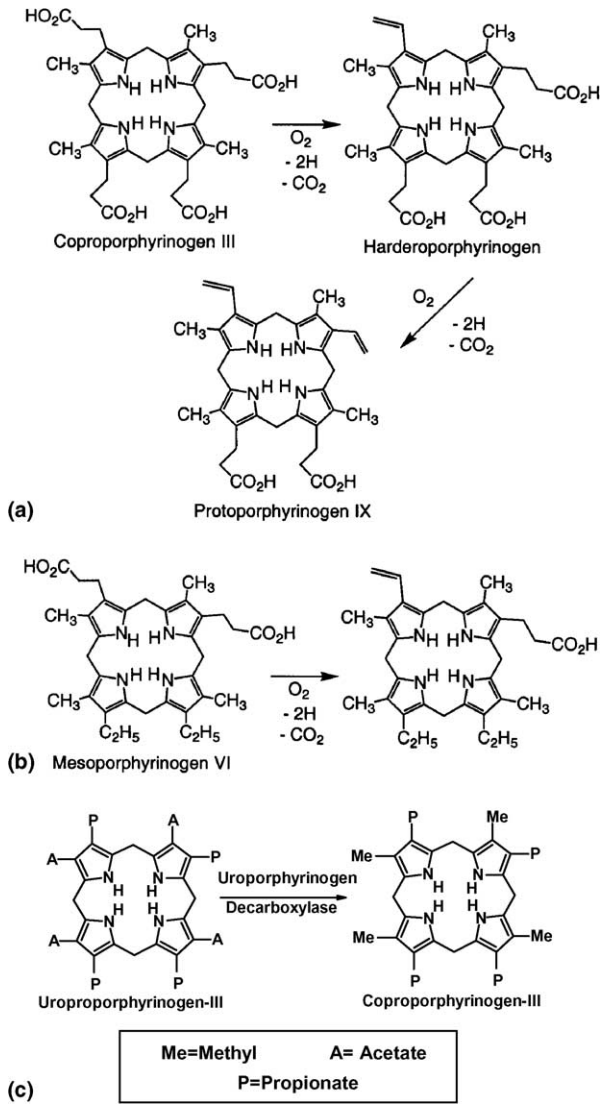

Figure 1. (a) Reaction catalyzed by coproporphyrinogen oxidase using coproporphyrinogen-III. (b) Reaction catalyzed by coproporphyrinogen oxidase using mesoporphyrinogen-VI. (c) Reaction catalyzed by uroporphyrinogen decarboxylase.

Coproporphyrinogen oxidase has been isolated from several species using a variety of purification strategies depending on the source of the enzyme. The sources of isolated copro'gen oxidase include rat liver,[11] *Saccharomyces cerevisiae*,[12] bovine liver,[13,14] yeast,[15] mouse liver,[16] barley and tobacco,[17] human placental library,[18] and *E. coli*.[19] As yet the chicken red blood cell enzyme has not been isolated. This is unfortunate since many of the substrate recognition studies have been done with this enzyme source.[2,20–23] One problem with using the chicken red blood cells as the enzyme source is that the vast majority of the protein in the incubations is hemoglobin. Also, Jackson et al.[24] showed that both URO-D and copro'gen oxidase can, in specific cases, use the same substrate, pentacarboxylate intermediate (penta dab) thus potentially leading to problems in kinetic evaluations when using chicken red blood cells. Therefore, goals of this research were to accomplish the separation of coproporphyrinogen oxidase from hemoglobin, as well as separation from uroporphyrinogen decarboxylase.

2. Methods

2.1. Enzyme preparation

The enzyme was prepared using the procedure described previously.[1,2] Chicken blood (approximately 100 mL per chicken) was obtained from chickens following decapitation. The chicken blood was collected into a beaker containing 10 mg heparin dissolved in 10 mL of 0.9% (w/v) NaCl for every 300 mL of chicken blood. Heparin was used to prevent the coagulation of the blood so that intact red blood cells (RBCs) could be isolated by centrifugation at 4000 rpm at 4 °C for 10 min using the JA-20 Beckman rotor. The RBCs were washed three times with 0.9% NaCl then lysed by osmotic shock using ice-cold deionized water. Then 47 mL of 0.6 M KCl was added for every 225 mL of original blood followed by gentle mixing. This preparation was designated as chicken blood hemolysates (CBH). Aliquots of 10 or 25 mL were transferred to polypropylene tubes, frozen on dry ice and stored at −80 °C until needed. Enzyme activity was monitored following the micro-assay procedure of Jones et al. using freshly prepared substrate.[25]

2.2. Development of chromatography resin

The porphyrin-coupled resin was processed following the procedure of Mukerji and Pimstone,[3] who reported an affinity chromatographic procedure using porphyrin covalently coupled to sepharose. They covalently attached uroporphyrin to ω-aminohexyl sepharose 4B using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. In this research, we used coproporphyrin-III (100 mg, obtained from Aldrich Chemical Company, Milwaukee, WI) as the ligand attached to the resin (50 mL) following their procedure. Approximately 28% of the total porphyrin was covalently attached to the resin. The porphyrin-resin had a pink appearance and exhibited fluorescence using UV light due to the covalently attached porphyrin; the unmodified ω-aminohexyl sepharose 4B was white and exhibited no fluorescence. The resin was poured into a 32 × 2 cm column and stored in buffer (10 mM potassium phosphate buffer, 10 mM dithiothreitol, pH 6.8), wrapped in foil to reduce exposure to light, in a cold room at 4 °C. Before use, the resin was washed with loading buffer (10 mM potassium phosphate buffer, pH 6.8) to equilibrate the column. While the resin was being washed, chicken blood hemolysate (60 mL) was thawed then centrifuged with the JA-17 rotor at 10,000 rpm for 20 min at 4 °C. The supernatant was collected, volume determined, and loaded onto the porphyrin-resin column. A sample was saved for protein and enzyme analysis. The flow rate for the column was controlled at 3 mL/min and 10 mL fraction volumes were collected. After the fifth fraction was collected, the eluting buffer (10 mM potassium phosphate, 0.8 M KCl, pH 6.8) was applied. Fractions were collected until a total of 15 fractions were obtained. Each fraction was evaluated for total protein,[26] number of proteins by

SDS-polyacrylamide gel electrophoresis,[27] and enzyme activities.[25] For determination of enzyme activity, uroporphyrinogen-III was used as substrate for detection of uroporphyrinogen decarboxylase and mesoporphyrinogen-VI (meso-VI; Fig. 1b) was used as substrate for detection of coproporphyrinogen oxidase. Use of the meso-VI as substrate was very beneficial since oxidative decarboxylation of this substrate by copro'gen oxidase results in a single monovinyl product that does not co-elute with protoporphyrin-IX in our HPLC system.[28] Since there are detectable amounts of endogenous protoporphyrin-IX in CBH and some fractions from the column, this meso-VI substrate allowed for unambiguous determination of enzyme activity. Enzyme activities are reported as % product formed in 20 min/mg protein in the incubation. As a control, underivitized ω-aminohexyl sepharose 4B was also loaded in a column and used under the same chromatographic conditions with fractions analyzed as with the porphyrin column. Chromatographic data are reported as mg protein yield and enzyme specific activity (% product/20 min/mg protein).

3. Results

Figure 2 shows the elution of protein from the coproporphyrin-III resin. Greater than 93% of the proteins from the column eluted before fraction eight. After collection of fraction 5, the buffer was changed to 10 mM potassium phosphate, 0.8 M KCl, pH 6.8. The initial experiments tested the ability of the ligand resin to separate copro'gen oxidase and the URO-D activity (Fig. 3). The peak activity of URO-D was found in fraction three while the peak copro'gen oxidase activity was found in fraction 10. Thus this chromatographic step results in a rapid and facile separation of these two enzymes.

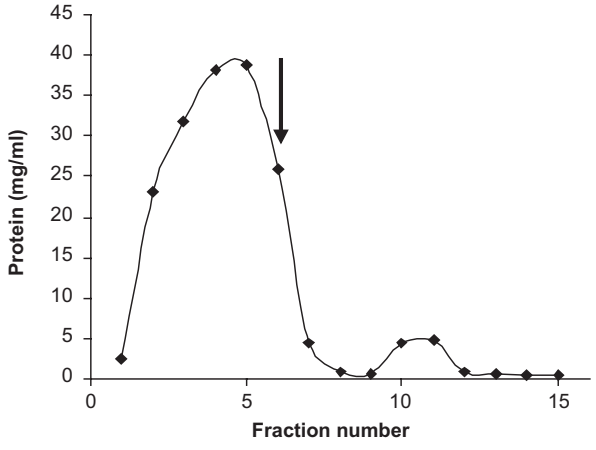

Figure 2. Elution of protein from coproporphyrin-III modified resin. Protein was evaluated by the method of Lowry et al.[26] and the *y*-axis is in units of mg protein per mL of fraction. Ten milliliters fractions were collected at a flow rate of 3 mL/min. The arrow indicates where the ionic strength was changed to 10 mM potassium phosphate, pH 6.8 with 0.8 M KCl.

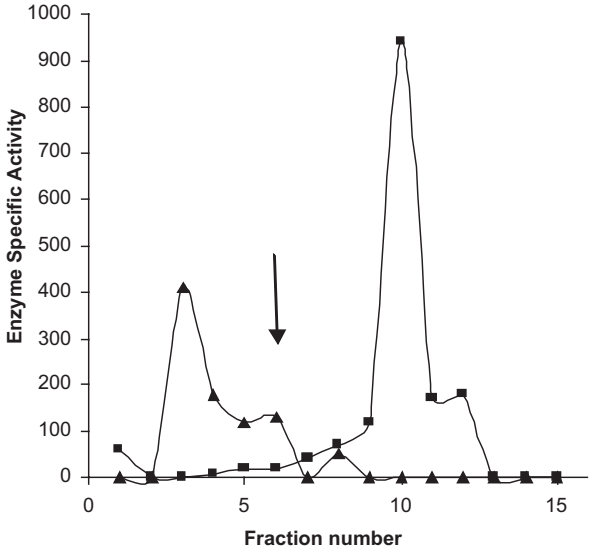

Figure 3. Elution of uroporphyrinogen decarboxylase activity and coproporphyrinogen oxidase activity from the coproporphyrin-III resin. Ten milliliters fractions were collected at a flow rate of 3 mL/min. The arrow indicates where the ionic strength was changed to 10 mM potassium phosphate, pH 6.8 with 0.8 M KCl. The specific activity for uroporphyrinogen decarboxylase is reported as % product per 20 min/mg protein (▲) while that for copro'gen oxidase is reported as % product formed per 20 min/mg protein $\times 10^3$ (■).

To see if a delay in the application of the eluting buffer would have any affect on the elution of copro'gen oxidase from the resin, the eluting buffer was applied after the collection of fraction 15. The results are shown in Figure 4. The peak in copro'gen oxidase activity was seen in fraction 20 for both the control and the porphyrin bound resins thus indicating a specific interaction between copro'gen oxidase and the resin. The majority of the protein applied to the column eluted before fraction 8.

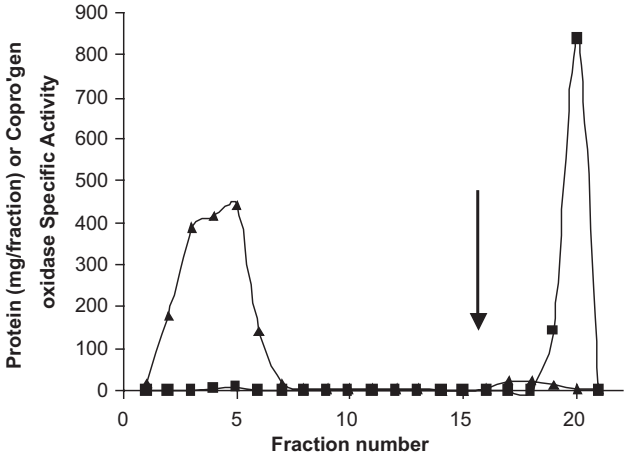

Figure 4. Normal elution of protein (▲) but retarded elution of copro'gen oxidase activity (■) from the coproporphyrin-III resin after delay in applying elution buffer containing 0.8 M KCl. The arrow indicates where the ionic strength was changed to 10 mM potassium phosphate, pH 6.8 with 0.8 M KCl.

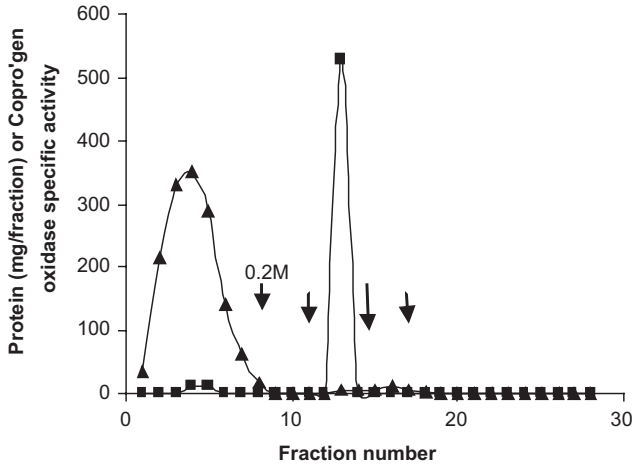

Figure 5. Use of a step gradient to test for selective elution from the coproporphyrin-III resin. Thirty milliliters of the eluting buffers of 0.2, 0.4, 0.6, and 0.8 M KCl were applied after the collection of fractions 8, 11, 14, and 17, respectively (as indicated by the arrows). Filled triangles indicate protein and filled squares indicate coproporphyrinogen oxidase activity.

To test if a buffer gradient would differentiate the copro'gen oxidase elution pattern from the two resins, a step gradient elution pattern was employed. The eluting buffer (of 100 mM sodium phosphate at pH 6.8) containing either 0.2, 0.4, 0.6, or 0.8 M KCl was applied after the collection of fractions 8, 11, 14, or 17, respectively. These results are shown in Figure 5. The peak in copro'gen oxidase activity was seen in fraction 13. This peak in copro'gen oxidase activity corresponds with the forefront of the first eluting buffer containing 0.2 M KCl. This fraction contained only 0.5% of the total protein applied to the column and thus represents a substantial purification step for this enzyme.

As a control, the experiment was repeated using untreated resin. The results (not shown) were essentially identical to those found using the porphyrin modified resin with the peak URO-D specific activity being found in fraction three while the peak activity of copro'gen was found in fraction 10. Again, almost all of the protein eluted before fraction eight indicating no retention by the resin. There was no difference in the elution profile from either resin used (data for unmodified resin not shown).

Analysis by SDS-PAGE was also used to evaluate proteins eluted in these experiments. The control and porphyrin-resin experiments showed no discernable differences in the number and apparent molecular weight of proteins detected in the electrophoretic gels (Fig. 6a and b for the ligand column). In the first eight fractions, the major eluting protein was hemoglobin. Fractions nine through 15 showed proteins other than hemoglobin. In fraction number 10, which had the highest activity of coproporphyrinogen oxidase, there were at least 11 protein bands evident. Thus, it is evident that copro'gen oxidase was not purified by this single chromatographic step.

4. Discussion

Using the porphyrin coupling conditions and column elution conditions of Mukerji and Pimstone,[3] the majority of the CBH supernatant proteins (approximately 93%) eluted in the first eight fractions (total of 80 mL eluted) from the column. Fraction three had the majority of the uroporphyrinogen decarboxylase activity while the copro'gen oxidase enzyme activity was found in fraction number 10. The elution profile of URO-D suggests no retention of this enzyme by the resin. This is in contrast with retention of copro'gen oxidase by the resin. Thus, the chromatographic step rapidly separates URO-D and copro'gen oxidase from each other without, however, purifying either enzyme. Using either resin, the elution of copro'gen oxidase was only accomplished after an increase in the ionic strength. It was initially assumed that the retention of copro'gen oxidase activity was due to specific interactions of the enzyme with the coproporphyrin-III molecule covalently attached to the resin. However, similar profiles for protein yield and enzyme activity were obtained for the unmodified column. This suggests that at least one noncovalent interaction occurred between coproporphyrinogen oxidase and the resin, that is, independent of the porphyrin. One noncovalent attractive interaction could be due to the resin linker, which is a primary amine group, and at pH 6.8 (the pH of the running and eluting buffers) the primary amine would be protonated and therefore has a net positive charge. When the eluting buffer is applied, ions from the eluting buffer could be disruptive of the ionic interactions that occur between the enzyme and the resin. The resin is not likely to be fractionating copro'gen oxidase by size because the fractionation range, according to manufacturer specifications, is from 60 to 20,000 kD (Sigma Chemical Company). Copro'gen oxidase has been purified from many other sources and molecular weights reported. Copro'gen oxidase purified from rat liver,[11] *S. cerevisiae*,[12] and bovine liver[13] had a molecular weight ranging from 72 to 80 kD in the monomeric form. Copro'gen oxidase purified from yeast,[14] mouse liver,[15] barley and tobacco,[16] bovine liver,[17] and human placental library[18] had a molecular weight ranging from 35 to 38.4 kD for this enzyme.

The SDS-PAGE analyses of each fraction indicated the same bands for corresponding fractions from both column resins. Independent of the retention mechanism, both resins clearly were able to affect a separation of uroporphyrinogen decarboxylase from coproporphyrinogen oxidase. This is of interest because coproporphyrinogen-III is the product produced from uroporphyrinogen decarboxylase, thus it was possible that the URO-D enzyme could also be retained on the column with coproporphyrinogen oxidase.

The SDS-PAGE indicated that the majority of the hemoglobin eluted in the first eight fractions. SDS-PAGE analysis showed that very little hemoglobin was detected in the fraction with the highest copro'gen oxidase enzyme activity. Since the most active fraction for copro'gen oxidase was number 10 in Figure 3, 93% of

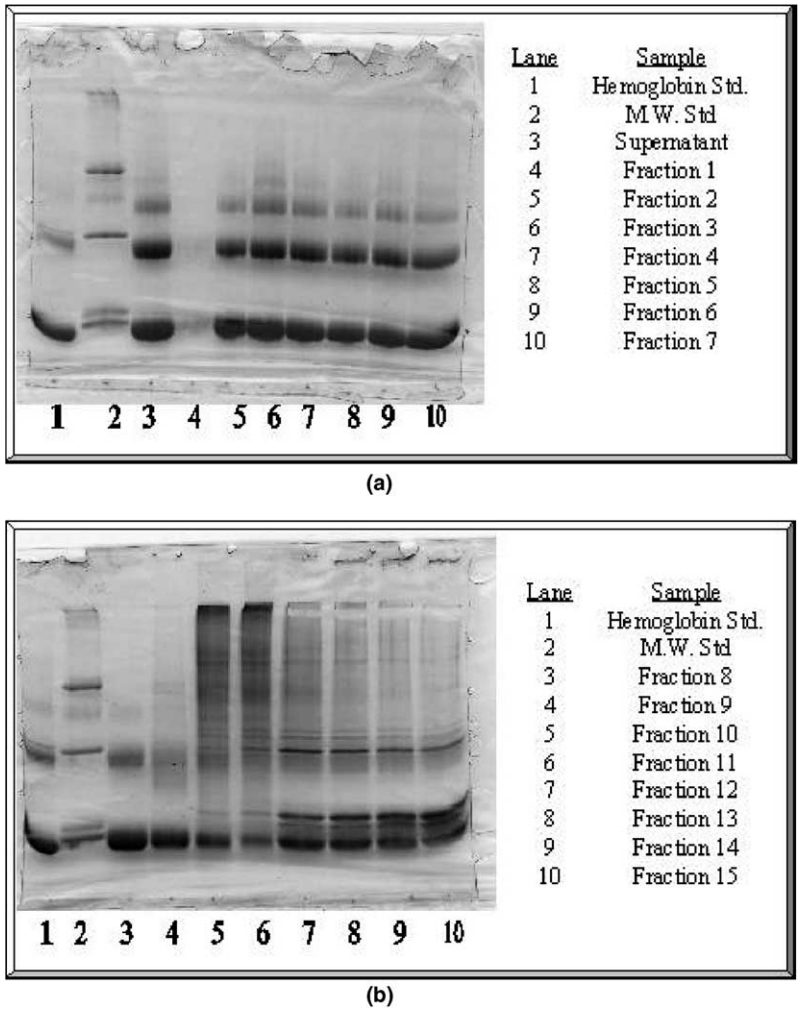

Figure 6. (a, b) SDS-PAGE of fractions from ligand column shown in Figure 3.

the protein was removed before copro'gen oxidase eluted from the column. Fraction 10 contained 2.5% of the eluting protein. The SDS-PAGE analysis of fraction 10 indicated that there were 11 proteins eluting with copro'gen oxidase. There was no one predominant band but some bands migrated with apparent molecular weights in the ranges previously reported for copro'gen oxidase. Thus, the enzyme is not purified by this single column step. It is, nevertheless, a substantial purification step able to remove some 97.5% of contaminating protein while retaining good enzyme activity. It was disappointing that this porphyrin resin procedure did not have the same success as that reported by Mukerji and Pimstone[3] using this ligand–resin strategy. Thus, their procedure appears to not have wide application. Further attempts to purify the copro'gen oxidase after the resin–ligand chromatography (using ion exchange chromatography, gel filtration chromatography, or ammonium sulfate precipitation) resulted in loss of enzyme activity.

Two positive achievements of the chromatographic procedure are that a majority of the hemoglobin, in addition to uroporphyrinogen decarboxylase, have been successfully separated from chicken RBC copro'gen oxidase. Separation of uroporphyrinogen decarboxylase from coproporphyrinogen oxidase was important since past kinetic studies to assess substrate selectivity by these enzymes were performed using CBH. Studies with either uroporphyrinogen decarboxylase or coproporphyrinogen oxidase can now be undertaken without interference from the other enzyme. Since CBH contains both porphyrinogen-processing enzymes, the past substrate selectivity studies may need careful re-examination.

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