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AFFINITY CHROMATOGRAPHY OF LYSYL HYDROXYLASE ON CONCANAVALIN A-AGAROSE

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Summary

Lysyl hydroxylase (peptidyllysine,2-oxoglutarate:oxygen 5-oxidoreductase, EC 1.14.11.4) has a high affinity for columns of concanavalin A-agarose, which was markedly reduced in the presence of α-methyl-D-mannoside, suggesting that the enzyme is a glycoprotein. Once bound, the enzyme could not be eluted with the glycoside alone, whereas an effective elution was achieved by a combination of α-methyl-D-mannoside and ethylene glycol. The data thus suggest that hydrophobic interaction stabilized the complex of the enzyme with the column. This information was applied to obtain a lysyl hydroxylase purification of about 3000-fold with a recovery of more than 10% from extract of chick embryos by relatively simple steps.

Lysyl hydroxylase (peptidyllysine,2-oxoglutarate:oxygen 5-oxidoreductase, EC 1.14.11.4) catalyzes the synthesis of hydroxylysine in collagen by hydroxylation of lysyl residues in peptide linkages (for recent reviews, see refs. 1—3). The reaction requires α-ketoglutarate, molecular oxygen, ferrous iron and a reducing agent, which can be ascorbate [4—9], the α-ketoglutarate being stoichiometrically decarboxylated during the reaction [10]. The enzyme has been partially purified and characterized from newborn rat skin [11] and chick embryos [8—10, 12—15], but has not been isolated as a homogeneous protein. The highest degree of purification, of up to 4000-fold, has been reported from chick embryo extract by a procedure consisting of eight conventional protein purification steps [12].

Lysyl hydroxylase has a high affinity for concanavalin A-agarose, but all attempts to elute the enzyme from this material have been unsuccessful [12]. Recent studies on human fibroblast interferon [16,17] and on chick

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and human collagen glucosyltransferase [18] indicate that hydrophobic interaction contributes to the affinity of these proteins for concanavalin A-agarose. In the present paper similar findings are reported on the elution of lysyl hydroxylase from convanavalin A-agarose, and an application of this information to obtain a relatively simple and effective purification of the enzyme is suggested.

The chick embryo ammonium sulphate enzyme used in these studies was obtained as a fraction of the 15 000 × *g* supernatant precipitated with 17—55% $(NH_4)_2SO_4$ saturation as described previously [12], except that the final dialysis was carried out against a solution (termed "enzyme buffer") containing 0.2 M NaCl, 0.1 M glycine, 10 mM $MnCl_2$, 10 μM dithiothreitol, 1% glycerol and 20 mM Tris·HCl buffer adjusted to pH 7.5 at 4°C. The enzyme was then diluted to a protein concentration of about 15 mg/ml, and passed through a column of concanavalin A-agarose (Concanavalin A-Sepharose 4-B, Pharmacia) equilibrated with the enzyme buffer. The column was eluted and the enzyme purified further as described in the legends to the figures. All procedures were carried out at 0—4°C.

Lysyl hydroxylase activity was assayed with [^{14}C]lysine-labelled protocollagen substrate as described previously [8].

The binding of lysyl hydroxylase to concanavalin A-agarose was tested with a column having a bed volume of 3 ml. When chick embryo ammonium sulphate enzyme with a protein concentration of 16 mg/ml was passed through the column, more than 90% of the protein was found in the column effluent. The enzyme activity of the column effluent per ml was less than 20% of that in one ml of the ammonium sulphate enzyme until about 45 ml of the enzyme had passed through the column (Fig. 1, •). When similar experiments were carried out with the addition of 0.3 M α-methyl-D-mannoside to the enzyme buffer, most of the enzyme was recovered in the column effluent (Fig. 1, ○) suggesting that the binding of lysyl hydroxylase to concanavalin

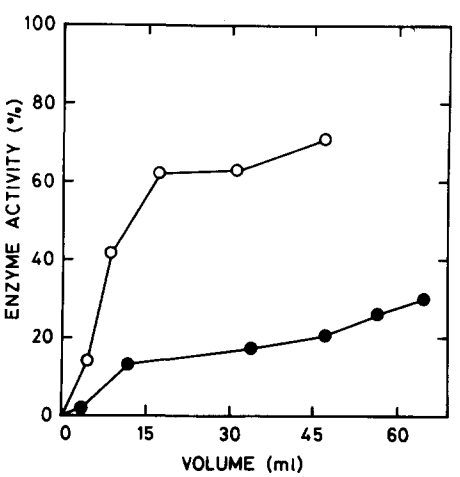

Fig. 1. Binding of lysyl hydroxylase to concanavalin A-agarose in the absence (•) or presence (○) of 0.3 M α-methyl-D-mannoside in the enzyme buffer. Chick embryo ammonium sulphate enzyme with a protein concentration of 16 mg/ml was passed through a 3-ml column with a flow rate of about 3 ml/h. The enzyme activity of the column effluent in ml is expressed as a percentage of that of the original ammonium sulphate enzyme.

A-agarose was largely due to the presence of some carbohydrate units in the enzyme molecule. It has recently been reported that an analogous enzyme, prolyl hydroxylase, is likewise a glycoprotein binding to concanavalin A [19].

Once lysyl hydroxylase had become bound to the column, it was possible to elute part of the protein from the column with enzyme buffer containing 1 M α-methyl-D-mannoside, there being no elution of enzyme activity (as shown for a 20-ml column in Fig. 2). The enzyme could then be eluted with enzyme buffer containing 0.3 M α-methyl-D-mannoside and 50% ethylene glycol (Fig. 2). It thus seems that hydrophobic interaction stabilized the complex of the enzyme with the column.

In some experiments the eluate from a 20-ml column was concentrated to about 10 ml by ultrafiltration in an Amicon ultrafiltration cell with a PM-30 membrane. This sample was applied to an 8% agarose gel column of size 2.5 × 90 cm. The enzyme activity was found as one peak, the elution position of which corresponded to a molecular weight of about 200 000 (Fig. 3).

Multiple forms of lysyl hydroxylase have previously been observed during purification from chick embryos, the elution positions of the two main forms corresponding to molecular weights of about 550 000 and 200 000 [8,12]. No evidence for multiple forms was found in the present study, suggesting that treatment of the enzyme with ethylene glycol converted the larger form to the smaller one.

Hydroxylysyl residues perform two important functions in collagen: They serve as sites of attachment for the carbohydrate units and participate in the interchain crosslink formation [2,20]. The significance of hydroxylysine in stabilizing the crosslinks is clearly demonstrated in one form of the Ehlers-Danlos syndrome, in which a deficiency in lysyl hydroxylase activity leads to a severe connective tissue disorder [21—23]. Some properties of the mutant

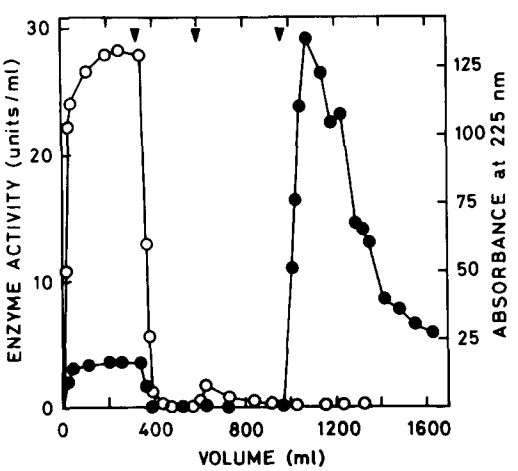

Fig. 2. Chromatography of lysyl hydroxylase on a 20-ml concanavalin A-agarose column. Chick embryo ammonium sulphate enzyme, 340 ml, with a protein concentration of 18 mg/ml, was passed through the column with a flow rate of about 20 ml/h. The enzyme was eluted first with the enzyme buffer (first arrow), then with the enzyme buffer containing 1 M α-methyl-D-mannoside (second arrow), and finally with the enzyme buffer containing 0.3 M α-methyl-D-mannoside and 50% (v/v) ethylene glycol (third arrow). •, Enzyme activity (for units, see Table I); ○, peptide absorbance at 225 nm.

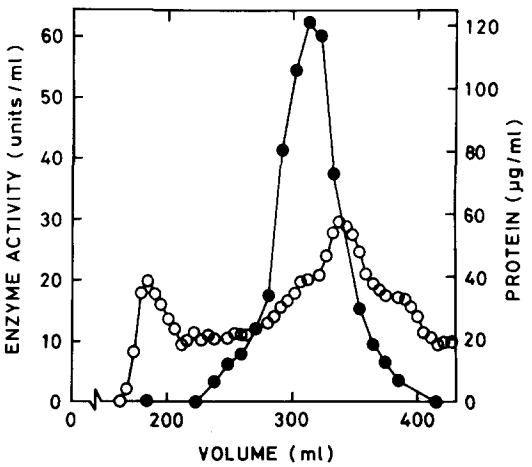

Fig. 3. Gel filtration of lysyl hydroxylase on an 8% agarose column. The eluate from a 20-ml concanavalin A-agarose column was concentrated as described in the text and applied to a Bio-Gel A 1.5-m column (200—400 mesh, Bio-Rad), 2.5 X 90 cm, which was equilibrated and eluted with the enzyme buffer without manganese. The fractions between 286 and 322 ml were pooled and constituted the purified enzyme as shown in Table I. •, Enzyme activity (for units see Table I); ○, protein.

enzyme in this disorder have been reported recently [24].

Although a number of studies have been carried out on lysyl hydroxylase, the enzyme has not been isolated as a homogeneous protein. This is to a large extent due to the tendency for the enzyme to lose activity with time, and after a purification of about 2400-fold by conventional procedures the recovery of enzyme activity was only about 0.4% [12]. Accordingly, affinity chromatography would seem ideal for the purification of this enzyme. However, attempts to develop procedures based on the affinity of the enzyme for its substrate have failed [12]. The present results indicate that chromatography on concanavalin A-agarose can be used as an effective step in the purification of lysyl hydroxylase, and with the procedure reported in this paper, a purification of about 3000-fold was obtained for lysyl hydroxylase from the 15 000 X *g* supernatant of chick embryo homogenate with a recovery of about 13% (Table I). The purification of another enzyme preparation was about 2800-fold with a similar recovery. Sodium dodecyl sulphate polyacrylamide gel electrophoresis [25] of the denatured and reduced protein in the

TABLE I

PURIFICATION OF LYSYL HYDROXYLASE FROM EXTRACT OF 14-DAY-OLD WHOLE CHICK EMBRYOS

One unit of enzyme activity is defined as the amount of enzyme present in 1 mg of the $(NH_4)_2SO_4$ fraction (17—55% saturation) obtained from the 15 000 X *g* supernatant of chick embryo homogenate [8,12].

Enzyme fraction	Total protein (mg)	Total activity (units)	Recovery (%)	Specific activity (units/mg)	Purification (-fold)
15 000 X *g* supernatant	32 000	16 000	100	0.5	1
$(NH_4)_2SO_4$ enzyme	12 800	12 800	80	1.0	2
Concanavalin A-agarose	9.1	4 110	26	452	904
Gel filtration	1.3	2 000	13	1540	3080

gel filtration fraction with highest specific activity showed the presence of two major and two minor bands, indicating that the enzyme was not pure. The molecular weights of the two major polypeptide chains were about 80 000 and 87 000, when studied using 5% polyacrylamide gel calibrated with standard polypeptide chains. The relative amounts of the two main chains varied in different gel filtration fractions, suggesting that they were not derived from the same protein, and only the chain with molecular weight about 87 000 always had its maximal concentration in the fraction with the highest enzyme activity.

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