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PREPARATION OF ANTIBODIES TO CHICK-EMBRYO GALACTOSYLHYDROXYLYSYL GLUCOSYLTRANSFERASE AND THEIR USE FOR AN IMMUNOLOGICAL CHARACTERIZATION OF THE ENZYME OF COLLAGEN SYNTHESIS

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Summary

Antibodies were prepared against chick-embryo galactosylhydroxylysyl glucosyltransferase and further purified by immunoaffinity chromatography. The antibodies gave a single precipitation line of identity by double immunodiffusion against crude or pure chick-embryo glucosyltransferase. The ability of the antibody to precipitate the transferase was not altered by destroying the secondary structure of the enzyme. The antibody also inhibited the enzyme activity. The degree of inhibition was higher with denatured citrate-soluble rat skin collagen as the substrate than with gelatinized rat skin insoluble collagen or free galactosylhydroxylysine.

The cross-reactivity of the glucosyltransferase between different species was low when studied by double immunodiffusion or inhibition kinetics. The antiserum showed no detectable cross-reactivity against other intracellular enzymes of collagen biosynthesis.

A line of complete identity was found in double immunodiffusion between the transferases from whole chick embryos and chick embryo tendon, kidney and cartilage. Inhibition by the antiserum of the enzyme from chick embryo tissues synthesizing different collagen types was relatively similar. The data do not support the hypothesis that galactosylhydroxylysyl glucosyltransferase has isoenzymes with markedly different specific activities or immunological properties.

Abbreviation IgG, immunoglobulin G

Introduction

The biosynthesis of the collagen molecule involves a number of post-translational modifications of the initial polypeptide chains. These include hydroxylation of appropriate prolyl or lysyl residues to 3-hydroxyprolyl, 4-hydroxyprolyl and hydroxylysyl residues and glycosylation of appropriate hydroxylysyl residues to galactosylhydroxylysyl and glucosylgalactosylhydroxylysyl residues. These reactions are catalyzed by five specific enzymes (for reviews, see Refs 1—5).

Galactosylhydroxylysyl glucosyltransferase catalyzes the synthesis of glucosylgalactosylhydroxylysyl residues by transferring the monosaccharide unit from UDP-glucose to galactosylhydroxylysyl residues. The reaction requires the presence of a bivalent cation, this requirement being best fulfilled by Mn^{2+} [6,7]. The enzyme has been isolated as a homogeneous protein from chick embryos and found to be glycoprotein [8—10] with a molecular weight of about 72 000—78 000, by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, consisting of only one polypeptide chain [8]. The enzyme has also been characterized by amino-acid analysis [10] and by kinetic studies on the reaction mechanism [11]

In the present study, an antiserum was prepared in rabbits against pure galactosylhydroxylysyl glucosyltransferase from chick embryos and the antibodies obtained were further purified using immunoaffinity chromatography. The antiserum was tested for its ability to inhibit the enzyme reaction with various substrates, and was used to examine whether distinct immunological differences in galactosylhydroxylysyl glucosyltransferase are found between different species and different chick-embryo tissues. The latter question was raised by the fact that marked differences are found in the extent of glucosylation of collagens between different tissues (see Ref. 3) and by recent demonstrations that a partial deficiency of galactosylhydroxylysyl glucosyltransferase activity in one family is associated with dominant epidermolysis bullosa simplex [12], a disease showing clinical symptoms only in the skin. An additional aspect for study was whether chick-embryo enzymes catalyzing other intracellular modifications in collagen biosynthesis show any cross-reactivity with the antiserum for the glucosyltransferase.

Materials and Methods

Materials UDP[^{14}C]glucose (227 Ci/mol) and UDP[^{14}C]galactose (274 Ci/mol) were purchased from New England Nuclear and non-radioactive UDP-glycosides from Sigma L-[U-^{14}C]proline (283 Ci/mol), and L-[U-^{14}C]lysine (345 Ci/mol) were from the Radiochemical Centre, Amersham

Denatured [^{14}C]proline-labelled and [^{14}C]lysine-labelled protocollagen substrates were prepared in freshly isolated chick-embryo tendon cells [13]. Citrate-soluble collagen substrate and gelatinized insoluble collagen were prepared from rat skin (see Ref. 14) and galactosylhydroxylysine was purified from sponge [15].

The immunoaffinity column was prepared by coupling pure galactosylhydroxylysyl glucosyltransferase to agarose by the CNBr-activation technique

[16] About 10 ml 4% agarose (Sepharose 4B, Pharmacia) was washed three times with an excess of distilled water, pH 11, after which about 2 g CNBr (Eastman), dissolved in acetonitrile, were added. The reaction was allowed to proceed for 15—20 min in an ice-bath with continuous stirring, the pH being maintained at 11 with 2 M NaOH. The mixture was then rapidly washed in a Buchner funnel with 50 ml of a solution of 0.5 M NaCl/0.1 M $NaHCO_3$, pH 8.3, and the agarose precipitate rapidly suspended in 5—10 ml of the same buffer. An aliquot of about 2 ml was transferred to a solution of 0.5 M NaCl/0.1 M $NaHCO_3$, pH 8.3/900 μg pure glucosyltransferase and the mixture was stirred gently at room temperature for 2 h, and at 4°C for 20 h. The gel was washed first with a large volume of 0.5 M NaCl/0.1 M $NaHCO_3$, pH 8.3, then with water, and finally with 0.5 M NaCl/0.1 M sodium acetate, pH 4 5. The gel was suspended in a buffer containing 0.15 M NaCl/50 mM Tris-HCl, pH adjusted to 7.5 at 4°C.

Assays for enzyme activities The assay of galactosylhydroxylysyl glucosyltransferase activity is based on measurement of the transfer of [^{14}C]glucose from UDP[^{14}C]glucose to galactosylhydroxylysyl residues in collagen. The samples were incubated for 45 min at 37°C in a final volume of 100 μl containing about 6 mg/ml heat-denatured citrate-soluble rat skin collagen or about 26 mg/ml gelatinized insoluble rat skin collagen as a substrate/2 mM $MnCl_2$/60 μM UDPglucose (8.1 Ci/mol)/1 mM dithiothreitol/50 mM Tris-HCl buffer, pH adjusted to 7.4 at 20°C. The reaction products were assayed as reported previously [17,18].

Hydroxylysyl galactosyltransferase [17], prolyl hydroxylase [19] and lysyl hydroxylase [20] activity were assayed as described elsewhere. The substrate in the assay of hydroxylysyl galactosyltransferase activity was gelatinized insoluble rat skin collagen, and that in the assay of prolyl and lysyl hydroxylase activity radioactively-labelled protocollagen (non-hydroxylated procollagen).

Preparation of antiserum against pure chick-embryo galactosylhydroxylysyl glucosyltransferase The enzyme was isolated as a homogeneous protein from chick embryos as described previously [8], and dialyzed exhaustively against 0.15 M NaCl before immunization. The enzyme solution (180 μg/ml) was mixed with an equal volume of complete Freund's adjuvant and injected intradermally at 10 sites in a rabbit's back. At a separate site, 0.5 ml crude *Bordatella pertussis* vaccine was injected subcutaneously. Further injections of 150 μg glucosyltransferase mixed with incomplete Freund's adjuvant were administered 2, 3 and 4 weeks later. The rabbit was bled at 1-week intervals by ear vein puncture beginning 2 weeks after the first injection. The sera were stored at −20°C until used.

Purification of antibodies. 10 ml antiserum were precipitated with $(NH_4)_2SO_4$ (0—40% saturation, 243 g/l). The $(NH_4)_2SO_4$ precipitate fraction was passed through a DEAE-cellulose under conditions described elsewhere [21], giving an IgG fraction of the antiserum. An aliquot of the IgG fraction was then passed at a flow rate of 0.5 ml/h through the immunoaffinity column (1 ml) equilibrated with 0.15 M NaCl/0.05 mM Tris-HCl buffer, pH 7.5 (4°C). The column was washed with 3 ml of a buffer containing 1% Triton X-100/0.5 M NaCl/50 mM Tris-HCl, pH adjusted to 7.5 at 4°C, and after that with 30 ml of a buffer containing 0.15 M NaCl/0.05 mM Tris-HCl, pH adjusted to 7.5 at

4°C. The column was eluted with 3 ml of 0.01 M potassium phosphate buffer, pH 6.8/3 M NaSCN, and the eluate was immediately passed through a Sephadex G-25 column equilibrated with 0.15 M NaCl. Fractions in the void volume were pooled and concentrated by ultrafiltration in an Amicon ultrafiltration cell with a PM-10 membrane.

Other assays Double immunodiffusion was carried out according to standard procedures on microscope slides covered with 1% agar in 0.02 M barbital buffer, pH 8.6. The slides were stained with Coomassie brilliant blue and the immunodiffusion result was examined both before and after staining. The protein content of the samples were assayed by peptide absorbance at 225 nm using bovine serum albumin as a standard.

Results

Characterization of the antiserum. The antiserum prepared in rabbits was tested for its ability to inhibit the glucosyltransferase activity. No inhibition was found for 3 weeks after the first injection, but a marked increase was evident thereafter (Fig. 1). About 1.5 μl of the antiserum was required to inhibit the activity of 0.7 munits of the enzyme by 50% under standard incubation conditions (Fig. 2). When galactosylhydroxylysine or gelatine was used as the substrate instead of the citrate-soluble collagen, the degree of inhibition was clearly less, and in the case of galactosylhydroxylysine an inhibition of 0.7 munits by 50% was achieved only with 5 μl antiserum (Fig. 2).

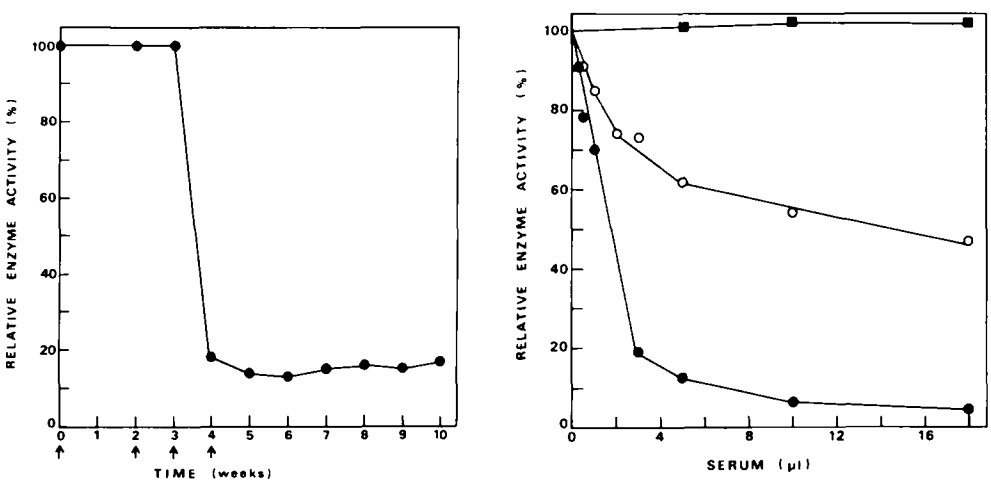

Fig 1 Formation of antibodies to chick-embryo galactosylhydroxylysyl glucosyltransferase in rabbits as a function of time Inhibition of chick-embryo galactosylhydroxylysyl glucosyltransferase activity by 2 μl antiserum was measured The assay system contained about 0 6 munits chick-embryo enzyme activity with citrate-soluble rat skin collagen as a substrate The arrows indicate the injections of antigen

Fig 2 Inhibition of galactosylhydroxylysyl glucosyltransferase activity (0 7 munits) by various amounts of non-immune serum (■———■), or antiserum, using citrate-soluble rat skin collagen (●———●) or galactosylhydroxylysine (○———○) as a substrate When galactosylhydroxylysine was used as a substrate, the reaction was stopped by adding 2 ml 1% phosphotungstic acid in 0 5 M HCl as usual but after centrifugation the supernatant of the sample was placed on a small column containing Dowex 50-X8 in H^+ form and treated as described previously [17]

The antiserum was also tested by double immunodiffusion, and a single precipitation line was seen. Similar results were obtained when the enzyme was heated for 10 min at 60°C before the test. A reaction of complete identity was obtained in immunodiffusion with pure and crude glucosyltransferase (Fig. 3).

Cross-reactivity of the antiserum with galactosylhydroxylysyl glucosyltransferase from different species The cross-reactivity of the chick-embryo enzyme antiserum was tested with galactosylhydroxylysyl glucosyltransferase from different species both by studying the inhibition of the enzyme activity and using immunodiffusion In the earlier experiments, the antiserum did not show any cross-reactivity with the enzyme from pig skin or human placenta, whereas a very slight cross-reactivity could be seen with the rat and mouse glucosyltransferase using this inhibition technique (Table I) Two additional experiments gave similar results (data not shown) In the immunodiffusion experiments a positive reaction was seen only with the chick embryo enzyme when the same amount of enzyme units from chick embryo, human placenta, pig skin and rat and mouse kidney cortex were tested However, when partially purified human glucosyltransferase was applied to the immunodiffusion in a 10-fold excess over the chick embryo enzyme (in units), a precipitation line showing identity with the chick-embryo glucosyltransferase was found

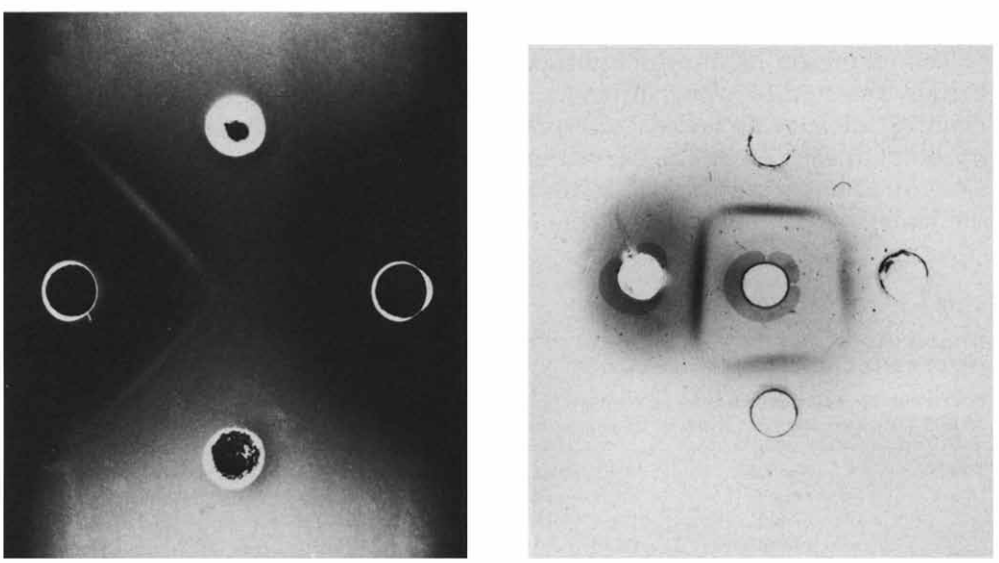

Fig 3 Double immunodiffusion analysis of chick-embryo crude (upper well) and pure (lower well) galactosylhydroxylysyl glucosyltransferase by antiserum (left well) prepared against pure chick-embryo galactosylhydroxylysyl glucosyltransferase The amount of crude enzyme applied corresponded to the activity of 4 2 munits under standard incubation conditions and that of pure enzyme to 85 6 munits The amount of antiserum used was 20 µl The right well was empty

Fig 4 Double immunodiffusion analysis of galactosylhydroxylysyl glucosyltransferase from various chick-embryo tissues The left well contained crude enzyme of whole chick embryos, the upper well that of tendon, the right well that of sternum, the lower well that of kidney and the centre well the antiserum (20 µl) The amount of tissue enzyme in each case correspond to an activity of about 2 6 munits under standard incubation conditions

TABLE I

INHIBITION OF GALACTOSYLHYDROXYLYSYL GLUCOSYLTRANSFERASE FROM DIFFERENT SPECIES BY ANTISERUM AGAINST THE CHICK-EMBRYO ENZYME

Gelatinized insoluble rat skin collagen was used as the substrate The amounts of the 15 000 × *g* supernatant of the tissue extracts were adjusted so that about the same amount of enzyme units were tested in each case 1 m unit of enzyme activity was defined as the activity required to synthesize an amount of the radioactive product (in dpm) corresponding to 1 nmol, in 1 h at 37°C, as described previously [18]

Source of enzyme	Activity found				
	without antiserum * (munits)	with 5 μl antiserum		with 10 μl antiserum	
		(munits)	(%)	(munits)	(%)
Whole chick embryos	0 49	0 27	(55)	0 14	(28)
Rat kidney cortex	0 49	0 48	(97)	0 45	(91)
Mouse kidney cortex	0 36	0 34	(93)	0 32	(91)
Human placenta	0 54	0 54	(100)	0 54	(100)
Pig skin	0 56	0 56	(100)	0 55	(98)

* 5 μl non-immune serum was used instead of antiserum

Cross-reactivity of the antiserum with galactosylhydroxylysyl glucosyltransferase from different chick-embryo tissues Crude preparations of the glucosyltransferase from whole chick embryo, chick embryo cartilage, chick embryo tendon and chick embryo kidney gave a reaction of complete identity when analyzed by double immunodiffusion (Fig. 4).

The inhibition of the glucosyltransferase activity from various chick-embryo tissues was studied using different amounts of the antiserum (Table II), the quantity of enzyme tested corresponding to about the same amount of activity units in each case. The enzyme from spleen and tendon required the lowest amount of antiserum and those from heart and skin the highest amount for a similar degree of inhibition, but the differences found were relatively small.

TABLE II

INHIBITION OF GALACTOSYLHYDROXYLYSYL GLUCOSYLTRANSFERASE ACTIVITY FROM DIFFERENT CHICK-EMBRYO TISSUES BY ANTISERUM

Citrate-soluble collagen was used as a substrate Enzymes from different tissues compared with were the 15 000 × *g* supernatants of tissue homogenates of 17-day-old chick embryos

Tissue	Enzyme activity tested (munits)	Amount of antiserum required for 50% inhibition * (μl)	Amount of antiserum required for inhibition of 1 munit enzyme activity (μl)
Spleen	0 87	0 59	0 678
Tendon	0 70	0 55	0 786
Kidney	0 71	0 65	0 915
Sternum	0 74	0 72	0 973
Lung	0 58	0 62	1 069
Heart	0 71	0 79	1 113
Skin	0 70	0 80	1 143

* Varying amounts of antiserum were added to the incubation mixture and the inhibition observed was plotted against the amount of antiserum used The amount of antiserum required for 50% inhibition was obtained from this curve

TABLE III

INHIBITION OF PROLYL HYDROXYLASE, LYSYL HYDROXYLASE AND HYDROXYLYSYL GALACTOSYLTRANSFERASE BY ANTISERUM PREPARED AGAINST GALACTOSYLHYDROXYLYSYL GLUCOSYLTRANSFERASE

The substrate for galactosylhydroxylysyl glucosyltransferase was citrate-soluble rat skin collagen The enzyme activities are expressed as relative values, taking the activity with non-immune serum as 100 The amount of glucosyltransferase and lysyl hydroxylase tested corresponded to about 40 ng pure enzyme protein and that pure prolyl hydroxylase was 20 ng The activity of the galactosyltransferase in munits was the same as that of the glucosyltransferase

Enzyme	Activity	
	with 5 μl antiserum (%)	with 10 μl antiserum (%)
Galactosylhydroxylysyl glucosyltransferase	18	8
Hydroxylysyl galactosyltransferase	100	100
Lysyl hydroxylase	98	96
Prolyl hydroxylase	100	99

Cross-reactivity of the antiserum with other intracellular enzymes of collagen synthesis The antiserum did not inhibit the activity of three other intracellular enzymes of collagen synthesis, prolyl 4-hydroxylase, lysyl hydroxylase and hydroxylysyl galactosyltransferase (Table III), when tested with 5 or 10 μl antiserum. All enzymes were prepared from chick embryos. Prolyl hydroxylase was shown to be a pure protein when examined by SDS-disc electrophoresis [19], lysyl hydroxylase was highly purified [22] and the glucosyltransferase and the galactosyltransferase were $(NH_4)_2SO_4$ fractions of the chick-embryo homogenate [17].

Purification of the antibodies The antibodies in the antiserum were further purified as shown in Table IV. A purification of about 40-fold was achieved with $(NH_4)_2SO_4$ precipitation, DEAE-cellulose chromatography and immunoaffinity chromatography on pure glucosyltransferase coupled to Sepharose 4B. The antibody yield was 6% when measured in terms of its ability to inhibit glucosyltransferase activity.

TABLE IV

PURIFICATION OF ANTIBODIES IN ANTISERUM PREPARED AGAINST CHICK-EMBRYO GALACTOSYLHYDROXYLYSYL GLUCOSYLTRANSFERASE

The amount of enzyme inhibited was 0 5 munits of the enzyme activity in a standard incubation condition Recovery is expressed as units of protein causing a 50% inhibition

Protein fraction	Total protein (mg)	Amount of protein required for 50% inhibition (μg)	Recovery (%)
Antiserum	63 3	94 5	100
IgG fraction	4.4	8 0	82
After immunoaffinity column and gel filtration	0.1	2 5	6

Discussion

Antibodies against galactosylhydroxylysyl glucosyltransferase were prepared here for the first time, and their specificity was ascertained by double immunodiffusion, in which an identical precipitation line was found both with crude and pure enzyme. The ability of the antibody to precipitate the enzyme was not altered when the enzyme was heated to destroy the secondary structure, suggesting that the antibody mainly identifies the primary structure. Comparison of the degrees of inhibition with different substrates indicates that the antiserum is more effective when tested with denatured citrate-soluble collagen than with gelatine consisting of relatively short pieces of partially degraded polypeptide chains or with free galactosylhydroxylysine It seems probable that the reaction of the antibody with the enzyme sterically prevents the binding of the substrate and that the binding site of the antibody is located in such a way that the enzyme reaction can take place at least partially in the presence of antibodies in the case of substrates of small molecular weight Similar findings have been reported on the inhibition of tadpole collagenase activity by its antiserum when tested with substrates of high and low molecular weight [23]

The cross-reactivity between galactosylhydroxylysyl glucosyltransferase from different species was found to be low when studied either by double immunodiffusion or by inhibition kinetics. Accordingly, it seems probable that the structure of the glucosyltransferase differs significantly from one species to another. Similar results have been found in the case of prolyl hydroxylase, in that only a weak cross-reactivity was observed between the enzyme from chick embryos and human tissues [24]. This contrasts with the cross-reactivity of collagen, which is very high between different species (see Ref. 25).

The antiserum prepared against the glucosyltransferase from chick embryos showed no detectable cross-reactivity against other intracellular enzymes of collagen biosynthesis from chick embryos in the inhibition assay.

Various chick-embryo tissues were found to contain an immunologically similar enzyme form, a line of complete identity being seen in double immunodiffusion between the enzyme from the whole embryo and the tendon, kidney and cartilage. Inhibition of galactosylhydroxylysyl glucosyltransferase tissues synthesizing different collagen types was relatively similar when compared with the marked differences found in glucosylgalactosylhydroxylysyl content between different collagen types Type I collagen from an adult animal contains 0.3 glucosylgalactosylhydroxylysyl residue/1000 amino acid residues, for example, in contrast to type IV collagen, which consists of 32 glucosylgalactosylhydroxylysyl residues/1000 amino acid residues (see Ref 3) The data obtained by these analyses do not support the hypothesis that tissues synthesizing genetically distinct collagen types have galactosylhydroxylysyl glucosyltransferase isoenzymes with markedly different specific activities or immunological properties. Nevertheless, small differences were found, and these may naturally be due to enzymes with slight differences in their properties, although they may also reflect the presence of small amounts of inactive enzyme or inhibitors in tissues such as the heart and skin. It should be noted that the results do not completely eliminate the possibility of a second glucosyltransferase (e.g., in kidney) having a very restricted substrate specificity

(type IV collagen) and unique antigenic properties.

The antibodies in the antiserum were purified using an immunoaffinity column in which pure glucosyltransferase was coupled to Sepharose 4B, and a purification of about 40-fold was achieved. The yield remained relatively low, however, which may be due to partial inactivation of the antibodies by thiocyanate, even though this was rapidly removed by gel filtration. Purified antibodies should make it possible to obtain further information on galactosylhydroxylysyl glucosyltransferase (e.g., concerning its localization in the cell) using immunoelectromicroscopy.

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