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Inhibition of β-Glucuronidase Activity by Albumin of Human Synovial Fluid (40358)

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In the course of a study on the kinetic properties of β-glucuronidase (EC 3.2.31) of human synovial fluid evidence was obtained indicating the presence of an endogenous inhibitor of this enzyme (1). The interest for this finding is enhanced by the fact that cartilage erosion in inflammatory joint diseases is considered to be caused by the degradative activity of various enzymes on the constituents of connective tissue (2, 3). According to current views the extent of this digestion should also depend on the level of specific inhibitors of the various degradative enzymes. An inhibitor of chondromucoprotein degrading enzyme(s) has been found in synovial fluids of patients with inflammatory joint disease (4). An inhibitor of collagenase, which is present in synovial fluids of rheumatoid arthritic patients (5, 6), has been found both in synovial fluid and serum (7). Furthermore, two inhibitors of proteinases, immunologically identical to serum α_1-antitrypsin (α_1-AT) and α_2-macroglobulin (α_2-M), have been detected in human synovial fluid (8).

The presence of an inhibitor of β-glucuronidase, an enzyme which participates in the catabolism of glycosaminoglycans in a concerted action with hyaluronidase (9), suggests that a large spectrum of enzyme activities are controlled in the extracellular compartments of connective tissue. This report describes a procedure for the purification of the inhibitor from synovial fluid and the analyses carried out to identify the compound.

Experimental procedures. Synovial fluid fractionation. Human synovial fluids were obtained from the knee joint of patients with various inflammatory and degenerative disease under aseptical conditions and frozen at −20°. The samples were thawed, freed of cellular elements by centrifugation, and digested with bacterial hyaluronidase (Miles-Servac, USA) as previously reported (1). After dialysis, synovial fluids were fractionated by gel filtration through a column of Sephadex G-200 (90 × 2.5 cm). Proteins were eluted with 20 m*M* Tris–HCl buffer, pH 8, containing 0.17 *M* NaCl and 10 m*M* $CaCl_2$, at a flow rate of 6 ml/hr. Fractions of two ml were collected, pooled (as indicated in the Results section), concentrated by ultrafiltration and examined for inhibitory activity. Further purification of the proteins with the lowest molecular weight was achieved by ion exchange chromatography in a column of DEAE A 52 (14 × 2 cm), equilibrated with 50 m*M* Tris-HCl buffer, pH 8. Elution with this buffer was followed by a NaCl gradient elution, at a flow rate of 24 ml/hr. Fractions of two ml were collected, pooled (as indicated in the Results sections), concentrated by ultrafiltration and tested for their inhibitory capacity of β-glucuronidase activity.

Enzyme assay. The inhibition of the β-glucuronidase activity by the synovial fluid and by the fractions isolated therefrom was routinely assayed by using the Helix pomatia enzyme (glusulase, ENDO, USA). The following commercial human serum albumins have been used in the studies of the inhibition of the enzyme: Human Albumin (95–100%), from Immuno-Oesterreiches Institut fuer Haemoderivative Ges.; Human Albumin (fatty acids free) from fraction V (SIGMA, USA). Occasionally, the extent of inhibition was also tested on a partially purified endogeneous β-glucuronidase. The enzyme assay (0.2 ml) was carried out with phenolphtalein-β-D-glucuronide as substrate (1).

Analytical procedures. Dialysis was performed first against the buffer solutions and then exaustively against deionized water. Ultrafiltration was performed using Amicon PM 30 membranes.

α_2-M and α_1-AT were quantitatively evaluated by single radial immunodiffusion using immunokits from Behringwerke. Proteins were determined by the method of Lowry *et*

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al. (10), by using bovine serum albumin (BSA) as standard.

Electrophoresis on cellulose acetate strips was carried out at 1.5 mA/cm for 20 min in Tris–Barbital buffer (Gelman Instrument Co., MI) pH 8.8 (μ = 0.06). Staining was performed by soaking the strips in a 5% TCA solution containing 0.5% of Ponceau-S stain (Gelman) for 20 min. Destaining was performed by soaking the strips in 5% TCA.

Electrophoresis for the immunoassays (2% agar) were performed in Tris–Barbital buffer pH 8.4 (μ = 0.06) for 50 minutes at 50 V and 6 mA. Rabbit total antiserum (50 μl) was incubated in the troughs at room temperature for 18 hr. At the end of the electrophoresis, the plates were washed for 8 hr with several changes of physiological solution and then dried over a blotting paper under a gentle stream of air for 2 hr. Staining was performed in methanol/5% acetic acid (10:90, v/v) containing azo-carmin G (Geigy). Destaining was accomplished by soaking the plates in 5% acetic acid.

Preparative polyacrylamide gel electrophoresis was carried out according to Sottocasa *et al.* (11).

Results. Gel filtration through Sephadex G-200 of human synovial fluids digested with hyaluronidase resulted in the separation of three peaks. α_2-M, synovial fluid β-glucuronidase and α_1-AT were recovered in peaks I, II, III respectively (Fig. 1). Inhibition of snail juice β-glucuronidase activity was exhibited only by the pooled fractions of peak III.

After concentration by ultrafiltration and extensive dialysis these fractions were applied to a column of DEAE A 52. The elution profile of this column is shown in Fig. 2. The small amount of protein eluted with Tris buffer did not show any inhibitory activity. The NaCl gradient separated a single peak, which contained the β-glucuronidase inhibitor and was devoid of any α_1-AT activity.

When compared to the synovial fluid and peak III of the gel filtration, the peak eluted from the DEAE column with the NaCl gradient (DEAE peak) exhibited a two-fold and four-fold increased inhibitory activity, respectively (Table I). It also showed a marked inhibition of the endogenous β-glucuronidase present in peak II of the gel filtration.

The DEAE peak was analyzed by electrophoresis on cellulose acetate. The electropherogram, stained for proteins, is shown in Fig. 3. A single protein band was observed,

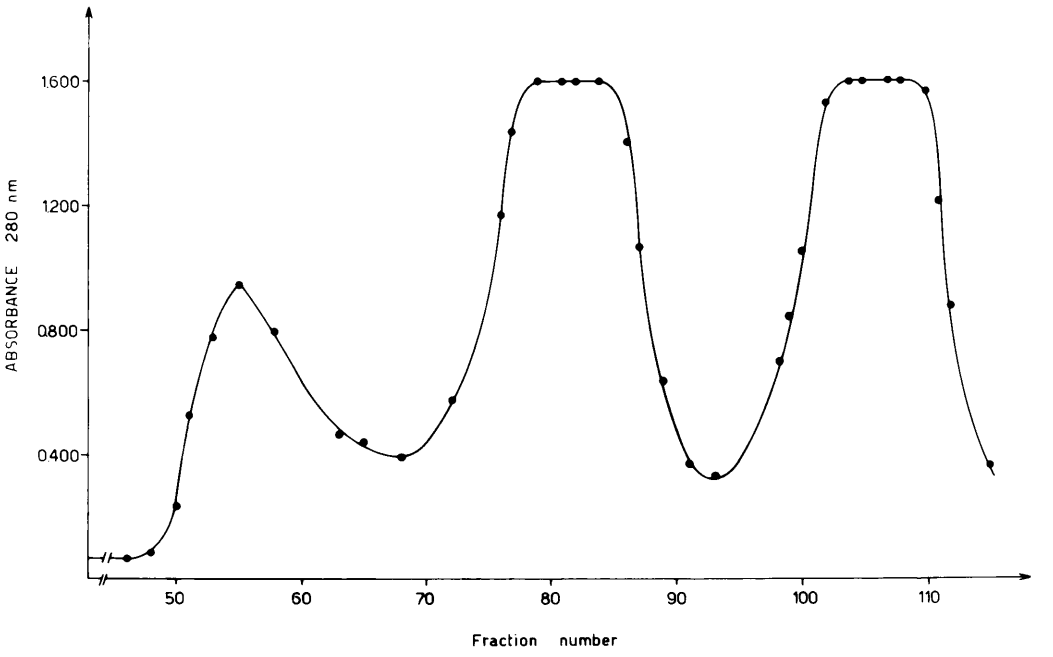

FIG. 1. Gel filtration of human synovial fluid on Sephadex G-200. Peak I = fractions 50–60; peak II = fractions 75–90; peak III = fractions 100–105. (For details, see Experimental Procedures).

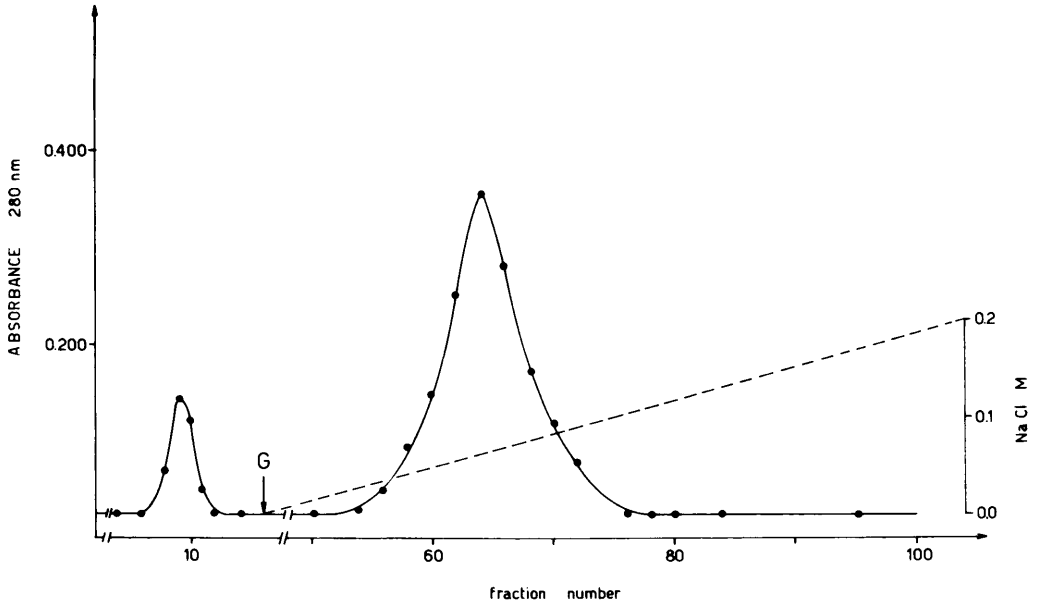

FIG. 2. Separation of the β-glucuronidase inhibitor by ion exchange chromatography on DEAE A 52. (For details, see Experimental Procedures).

TABLE I. INHIBITION OF β-GLUCURONIDASE BY PROTEIN FRACTIONS DERIVED FROM HUMAN SYNOVIAL FLUID.

Purification step	mg of protein/assay giving 50% inhibition
Synovial fluid	2.3
Peak III of Sephadex G-200	1.1
DEAE A 52 (NaCl gradient elution)	0.56

which migrated to a position corresponding to albumin of a serum sample analyzed in a parallel run.

By immunoelectrophoresis, the DEAE peak reacted as serum albumin (Fig. 4), giving a single symmetrical precipitin arc with rabbit antiserum to human serum.

By preparative polyacrylamide gel electrophoresis, the DEAE peak provided five subfractions (Fig. 5). Each subfraction inhibited the β-glucuronidase activity and reacted as serum albumin when tested by immunoelectrophoresis. In order to further demonstrate that albumin is the true inhibitor, we have tested also two commercial purified preparations of the compound as illustrated by Fig. 6. From the figure it appears that both preparations inhibit β-glucuronidase activity.

Discussion. Previous studies (1) have shown that the synovial fluid contains an inhibitor of β-glucuronidase, which exerts a competitive type of inhibition on the activity of both snail juice and rat liver enzyme. This inhibitor has now been purified and shown to be the albumin present in synovial fluid. The identification of albumin as the inhibitory substance is based on a comparison between the purified inhibitor and human serum albumin carried out by electrophoretic and immunologic techniques.

Albumin is known for its capacity of binding a number of small molecules. Hence, the inhibition of β-glucuronidase could be due to one such molecule and not to the protein itself. This possibility seems, however, unlikely since we have previously shown that a protease treatment of synovial fluid completely abolishes the inhibitory activity (1). Furthermore the results obtained by subjecting the inhibitor to the polyacrylamide gel electrophoresis indicate that the protein dissociate into five subfractions: each one, however, reacts with antibody to human serum albumin and inhibits β-glucuronidase. The fact that human albumin may be eterogeneous in purified preparations and in the serum itself has been already reported in literature (12). This microeterogeneity of hu-

man serum albumin may be directly transferred to the albumin of human synovial fluid, since plasma proteins reach the synovial space by diffusion (13). It appears therefore that inhibition of β-glucuronidase activity is shown also by the purest fractions of human albumin as those obtained by gel electrophoresis.

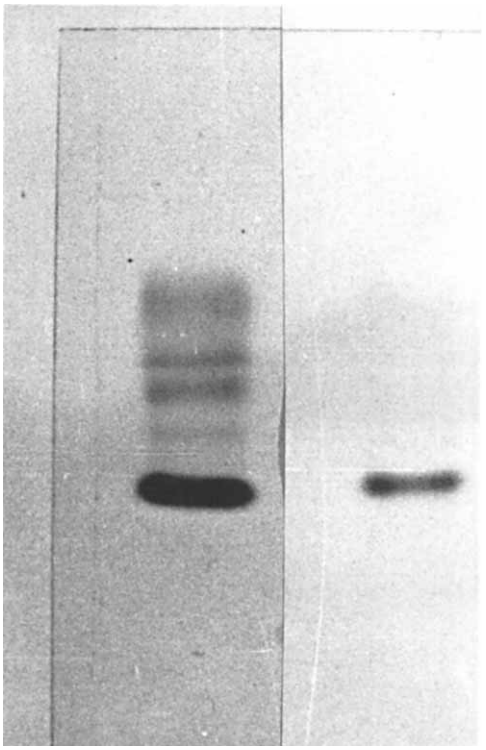

FIG. 3. Electrophoresis on cellulose acetate of serum proteins (left) and of combined fractions of the peak eluted from DEAE column (right).

Preparations of β-glucuronidase of high specific activity are stabilized in the assay by additions of 0.01% bovine serum albumin (14). This protective effect of albumin is apparently in contrast with our finding. One has, however, to consider that albumin of synovial fluid exhibits a competitive inhibition, which might not be seen in the usual assay conditions. However, in our experimental conditions, also commercial preparations of human serum albumin have been shown to be inhibitors of the enzyme activity (Fig. 6). This fact is of special interest since it has been reported (15) that commercial serum albumin preparations, usually stored for various periods of time by the manufacturing supply houses, may undergo alterations during storage, which might affect the biological properties of albumin in metabolic studies.

The human blood serum contains a number of high-molecular weight components, which inhibit hydrolytic enzymes such as collagenase, proteinases and other degrading enzymes (16–18). The demonstration that albumin can inhibit synovial fluid β-glucuron-

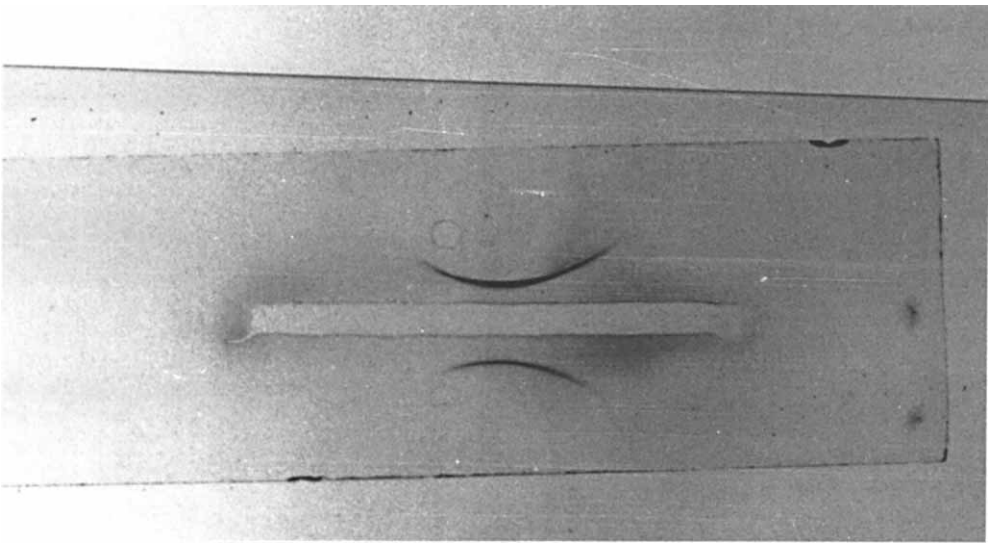

FIG. 4. Immunoelectrophoresis of combined fractions of the peak eluted from DEAE column (upper precipitation arc) and of serum albumin (lower arc). Antibody trough contained antiserum to whole serum proteins.

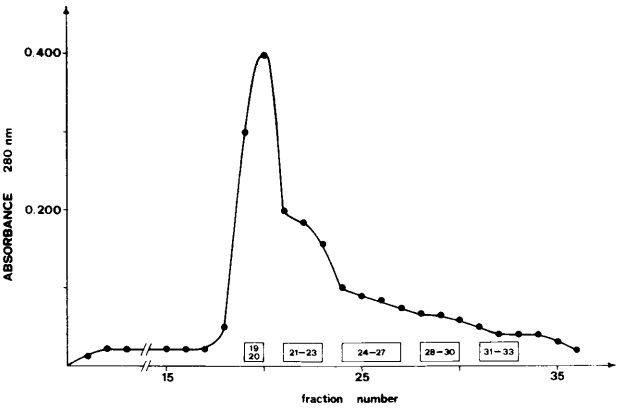

FIG. 5. Preparative polyacrylamide gel electrophoresis of combined fractions of the peak eluted from DEAE column.

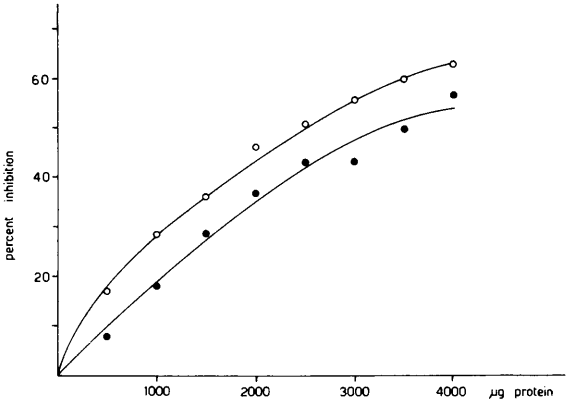

FIG. 6. Inhibition of snail juice β-glucuronidase by: ○ = human albumin from SIGMA; ● = human albumin from Immuno Oesterreiches Institut.

idase suggests that the serum proteins released into the inflammatory fluid can modulate a wide spectrum of degenerative reactions.

Summary. From human synovial fluid a protein inhibiting β-glucuronidase activity has been extracted and purified. The inhibitor is shown to be the albumin present in the synovial fluid. The identification of albumin is based upon a comparison between the purified inhibitor and human serum albumin carried out by electrophoretic and immunologic techniques.

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