Biochemical Pharmacology, Vol. 25, pp. 1507–1511. Pergamon Press, 1976. Printed in Great Britain.

HUMAN SERUM AND SYNOVIAL FLUID HYALURONIDASE—BOVINE TESTICULAR HYALURONIDASE IS NOT A VALID SUBSTITUTE IN DRUG EVALUATION STUDIES

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(*Received* 23 *September* 1975; *accepted* 10 *December* 1975)

Abstract—The properties of human serum and synovial fluid hyaluronidases were compared with each other and bovine testicular hyaluronidase. The parameters measured were pH optimum, molecular size, action on high molecular weight hyaluronate, substrate specificity, inhibition by non-substrate glycosaminoglycans and inhibition by sulphydryl reagents. The results obtained provide evidence that serum and synovial fluid hyaluronidases are similar, if not the same, enzyme. Serum and synovial fluid gave parallel results for all the parameters examined and differed substantially from those obtained with testicular enzyme. In particular, the sulphydryl properties of the human enzyme may offer a useful approach to the design of inhibitory drugs useful in the treatment of rheumatoid arthritis.

Markedly increased levels of lysosomal enzymes have been demonstrated in synovial fluid from inflamed joints in rheumatoid arthritis [1–5]. Both acid proteases and glycosidases are elevated, and have been implicated in the degradation of synovial hyaluronate [6] and cartilage proteoglycan [7].

The precise identity and specificity of the enzymes responsible has not been demonstrated. The existence of hyaluronidase in synovial fluid was originally postulated on the basis of the decreased viscosity of rheumatoid fluids [6]. Its presence was subsequently doubted [8] and its identity has remained obscure.

Studies from these laboratories have established the existence of a true hyaluronidase in synovial fluid [9]. Measurements of activity in rheumatoid fluid suggest it is not increased. Further, no significant difference could be found in the levels in supernatants of aspirates from osteoarthritic and rheumatoid knee joints. If synovial fluid hyaluronidase comes from the blood, then this would explain a synovial fluid level which remains largely independent of localised inflammatory conditions prevailing in joint tissues.

The present study compares the properties of human synovial fluid and serum hyaluronidase in an attempt to elucidate the origin of this enzyme and explain the lack of correlation of enzyme activity with inflammatory indices. As testicular hyaluronidase has been used by many workers [10–13] as a model system for examination the effects of drugs and other agents on postulated human hyaluronidases, it was considered desirable to include this enzyme in the present study to illustrate its differences to the human varieties.

MATERIALS AND METHODS

Bovine testicular hyaluronidase type IV (750 NF units/mg) and human umbilical cord hyaluronate type IIIS were obtained from Sigma Chemical Co., St. Louis, Mo. Chondroitin-6-sulphate (super special grade, from shark cartilage) was from Seikagaku Kogyo Co. Ltd., Tokyo. Beaded agarose gel of exclusion limit 500,000 mol. wt units (Biogel A 0.5 m), and hydroxylapatite (HTP) gel were obtained from Bio-Rad Laboratories, Richmond, California. Sodium gold thiomalate (Myocrisin for injection) was from May & Baker (Aust.) Pty. Ltd., and penicillamine hydrochloride from Dista Products Ltd., Liverpool, U.K.

Fluids were obtained fresh from clinics. The cells were centrifuged down and the supernatant stored frozen. Synovial fluid supernatant was used untreated or purified [9] by ammonium sulphate fractionation and agarose gel permeation chromatography, as indicated. Further purification of synovial fluid hyaluronidase was possible using 30 mM phosphate buffer (pH 7.0) on hydroxylapatite gel, but this gave low yields. Under these conditions, the enzyme did not adsorb to the gel, but a significant amount of other proteins did. At 20 mM phosphate or less, enzyme activity was not recovered in either the first bed volume or by elution with 0.3 M phosphate. The pooled fractions from agarose columns were free of hyaluronate and did not contain β-glucuronidase or β-*N*-acetylglucosaminidase (assayed as in [9]).

Serum was prepared from blood taken from healthy volunteers and without the use of anticoagulants.

Assays of hyaluronidase were carried out as previously described [9], but substituting the citrate–phosphate buffer of McIlvaine [14] for formate. No further additions of metal cations (e.g. as NaCl) were made to the incubation mixture. The assay measures the *N*-acetylglucosamine end groups produced from 200 μg of human umbilical cord hyaluronate at pH 4.0 and 37° in 20 hr.

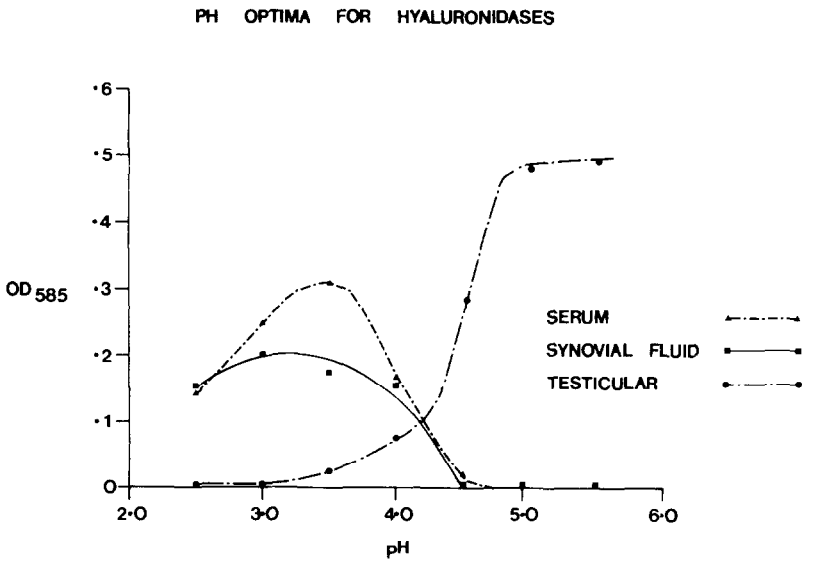

Fig. 1. pH Optima of hyaluronidase. The pH's shown were obtained by varying the proportions of 0.1 M citrate and 0.2 M disodium phosphate in the buffer.

When inhibitors were used, these were added to the normal enzyme incubation as solutions in buffer; control tubes for inhibitor studies were treated with inhibitor immediately before assay of *N*-acetylglucosamine, i.e. after the enzyme incubation.

RESULTS

pH Optima. The citrate phosphate buffer enabled a pH range of 2·5–5.5 to be studied, and suitable concentrations of the three sources of enzyme were chosen to give comparable activities. A distinctly different pH/activity function was obtained for bovine testicular enzyme compared to the other two (Fig. 1).

Serum and synovial fluid gave essentially the same pH/activity relationship, and behaved as typical lysosomal acid hydrolases. Their pH optimum was approximately 3.4, and no activity was detectable at or above pH 5.0. However, bovine testicular hyaluronidase had optimal activity at pH 5.0, and very low activity at pH 3.4.

Molecular size. The molecular size of the three enzymes was compared directly using agarose gel permeation chromatography with 0.05 M Tris–HCl, 0.15 M NaCl (pH 7.3) as eluting buffer. The bovine testicular enzyme has a well-characterised mol. wt of 60,000 [15]. The serum and synovial fluid enzymes were eluted slightly later than the testicular enzyme, indicating they have a lower mol. wt (Table 1).

Table 1. Elution ratios of hyaluronidases on agarose gel

Enzyme source	*Ve/Vo*
Human serum	1.84 ± 0.04
Human synovial fluid	1.83 ± 0.05
Bovine testicular extracts	1.72 ± 0.02

Enzyme samples containing blue dextran were applied to an analytical column (80 × 2.1 cm) of agarose 0.5 m gel (Bio-Rad) equilibrated with 50 mM Tris–HCl, 0.15 M NaCl (pH 7.3). Synovial fluid hyaluronidase was partially purified prior to chromatography by ammonium sulphate fractionation of synovial fluid (see [9]).

Cleavage of high molecular weight hyaluronate. The production of oligosaccharides from high mol. wt. hyaluronate was studied using column chromatography on Sephadex G75 (exclusion limit 50,000 mol. wt for dextrans). Size changes in the hyaluronate were investigated after hydrolysis by 1 N HCl at 100° for 1 hr, and after digestion for 30 hr at 37° with 10% human serum in 0.1 M formate pH 4.0 containing 80 μM saccharo-1,4-lactone. The latter concentration of saccharo-1,4-lactone is sufficient to give complete inhibition of β-glucuronidase [9], and since β-*N*-acetylglucosaminidase acts in concert with this enzyme [20], both exoglycosidases are effectively inhibited. Oligosaccharides which may be produced by a true hyaluronidase under these conditions will then be evident on column chromatography. The dilution of serum used (1:10) is sufficient largely to overcome the effect of the well-known serum inhibitor of hyaluronidase [16].

Incomplete acid hydrolysis also produces a mixture of oligosaccharides, and this was used for comparison. Extensive studies with testicular hyaluronidase [17] have already demonstrated the characteristic mixture of oligosaccharides produced by this enzyme.

Untreated umbilical cord hyaluronate (Fig. 2A) was completely excluded by the Sephadex G75, consistent with its high mol. wt. After hydrolysis with 1 N HCl, it gave a strongly retarded peak (Fig. 2B) which was totally dialysable. Incubation of hyaluronate with serum also produced oligosaccharides strongly retarded on G75 (Fig. 2C); this peak was partially dialysable.

Degradation could be routinely followed by the assay of *N*-acetylglucosamine end groups [18]: with serum and with synovial fluid supernatant, a time-dependent release of reducing ends was observed.

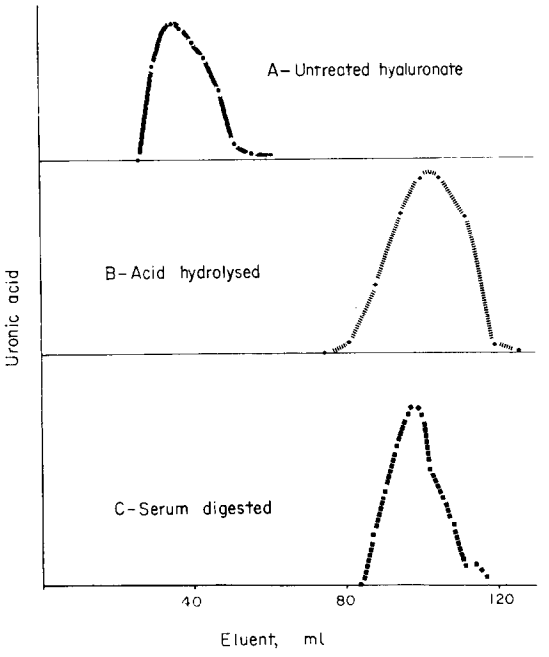

Fig. 2. Human umbilical cord hyaluronate was treated as shown and chromatographed on a Sephadex G75 column (1.6 × 70 cm) with 0.05 M Tris HCl, 0.15 M NaCl (pH 7.3) eluting buffer. Uronic acid was measured by the method of Bitter and Muir [29]. Acid hydrolysis (B) was with 1 N HCl at 100° for 1 hr; serum digestion (C) used 10% serum in formate buffer (pH 4.0) at 37° for 30 hr.

Substrate specificity. Insufficient synovial fluid hyaluronidase activity was obtained in purified preparations to permit measurements of substrate affinity constants. However, these preparations were shown to be active against both hyaluronic acid and chondroitin-6-sulphate. It was not meaningful to measure these constants with unpurified synovial fluid due to its endogenous hyaluronate content.

Constants were readily obtained, however, for serum and testicular enzymes. Notable differences were found between these enzymes in their affinity for hyaluronic acid and chondroitin-6-sulphate. Serum type enzyme had a considerably greater affinity for chondroitin-6-sulphate compared to testicular enzyme, as shown by the ratios in Table 2.

Inhibition by non-substrate glycosaminoglycans. The difference between bovine testicular enzyme and the other two enzymes was further demonstrated by their behaviour with non-substrate glycosaminoglycans whcih bear negative charges. Heparin was a considerably more potent inhibitor of testicular enzyme than dermatan sulphate, but these two polyanions gave similar inhibition with serum hyaluronidase and purified synovial hyaluronidase (Table 3).

Table 2. Substrate affinities of hyaluronidases

Enzyme source	Hyaluronate (μg/ml)	Chondroitin-6-sulphate (μg/ml)	Ratio
Human serum	800	1450	1.81
Bovine testicular serum	1250	3300	2.64

The concentration of glycosaminoglycan substrate in the normal assay was varied and the affinity constants derived from double reciprocal plots.

Table 3. Inhibition by heparin and dermatan sulphate

Enzyme source	Heparin	Dermatan sulphate	Ratio
Human serum	19	23	0.83
Purified synovial fluid*	22	27	0.82
Bovine testicular extract	65	14	4.64

Figures are per cent inhibition compared to controls with inhibitor added after enzyme incubation. Inhibitors were added to enzyme assays at a final concentration of 100 μg/ml.

* Purified synovial fluid hyaluronidase was the pool from agarose chromatography of an ammonium sulphate fraction of synovial fluid.

Thiol reagents and inhibition. Iodoacetamide and *p*-hydroxymercuribenzoate were used as specific thiol blocking agents with each of the three enzymes. Iodoacetamide appeared to have little, if any, effect on all three (Table 4). *p*-Hydroxymercuribenzoate, however, when used at a concentration of 1 mM, was a potent inhibitor of serum and synovial fluid enzymes. This concentration had no detectable effect on the activity of bovine testicular enzyme.

Cysteine was used to test the effect of a mild reducing agent. Under the conditions of the enzyme assays (pH 4 and pH 5) cysteine did not bring about non-enzymic degradation of hyaluronate. All three sources of hyaluronidase were activated by the addition of cysteine, the effect being least with the testicular preparation. Penicillamine showed a similar effect.

Sodium gold thiomalate (Myocrisin) has previously been reported as an inhibitor of lysosomal exoglycosidases [19], and we have also found it to be an inhibitor of synovial fluid hyaluronidase [25]. It inhibits serum and synovial fluid enzymes more strongly than testicular hyaluronidase (Table 4).

DISCUSSION

The present and previous studies [9] have now confirmed the presence of a true endoglycosidase, hyaluronidase, in synovial fluid. This hyaluronidase, alone, can attack high mol wt hyaluronate in synovial fluid. Exoglycosidases may, indeed, contribute to degradation, but only by acting on oligosaccharides

Table 4. Thiol reagents and inhibition

Drug	Serum	Synovial fluid	Testicular
2 mM Iodoacetamide	92	100	93
1 mM PCMB	0	0	100
2 mM Cysteine	170	170	150
2 mM Sodium gold thiomalate	<10	<10	25

Figures are per cent activity of controls with drug added after enzyme incubation.

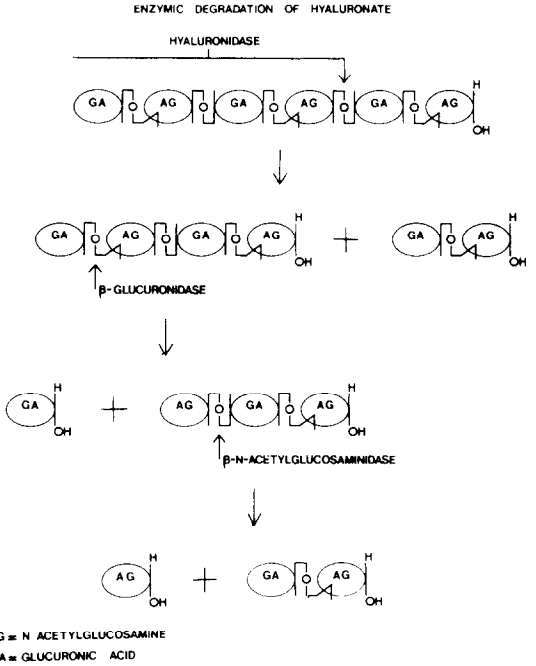

Fig. 3.

produced initially by the hyaluronidase (Fig. 3). In this way, they may act to ensure continued degradation by hyaluronidase, preventing the accumulation of inhibitory oligosaccharides [23, 30] in the reaction.

The interesting effects of electrolyte concentration on the concerted action of the exoglycosidases, β-glucuronidase and β-*N*-acetylglucosaminidase, and on the action of testicular hyaluronidase have been recently detailed [20, 21]. At physiological electrolyte concentration (and, by inference, in McIlvaine buffer) it may be concluded that exoglycosidase action is limited to small oligosaccharides. Testicular hyaluronidase is optimally active with high mol. wt hyaluronate at 0.2 M NaCl, and inhibition occurs above this concentration with both hyaluronate and oligosaccharides as substrates [21]. In our experience, serum and synovial fluid [9] hyaluronidase are inhibited by 0.3 M NaCl (using 0.1 M formate as assay buffer), the extent of inhibition varying with the purity of the preparations. Serum hyaluronidase purified by hydroxylapatite, ammonium sulphate and Sephadex G150 was inhibited 38% (unpublished data).

The earliest reliable chemical measurements of synovial fluid hyaluronate degrading activity [24] did not exclude the possibility that a combination of exoglycosidase activities could degrade high mol. wt hyaluronate without the presence of any real hyaluronidase. Viscometric assays were even less specific, as protease activity was included.

The results obtained provide evidence that serum and synovial fluid hyaluronidases are similar, if not the same, enzyme. Serum and synovial fluid gave parallel results for all the parameters examined, and differed substantially from those obtained with the testicular enzyme. Molecular size, pH optima, substrate specificity, polyanion inhibition, thiol properties and drug inhibition combined to present a strong body of evidence.

Since hyaluronidase levels in rheumatoid synovial fluids do not sugnificantly differ from those in osteoarthritic fluids [9], it is unlikely that the enzyme is produced locally in the joint by inflammatory or synovial cells. Further, the level of hyaluronidase in the serum of rheumatoid patients was found to be from two to ten times the level in the corresponding synovial fluid [25]. Thus a concentration gradient exists across the synovial membrane. Since serum and synovial fluid hyaluronidase have a mol. wt comparable with serum albumin, and the latter diffuses readily across the synovial membrane, it is probable that synovial fluid hyaluronidase is derived by diffusion from the blood. The permeability of the synovial membrane would then determine the level found in rheumatoid fluid, rather than the presence of the disease.

In pathological conditions of increased turnover by degradation, it is highly relevant to consider the interaction of these enzymes with drugs. Bovine testicular enzyme is the best known of the different types of hyaluronidase [26], and is used extensively in molecular structure and histochemical investigations. Its substrate requirements, products of reaction, inhibitors and physical structure have been reported previously [11, 15, 17].

Many investigators have assumed that testicular hyaluronidase behaves in the same way as any hypothetical hyaluronidase produced by tumours or rheumatoid joints [10, 12]. It has been postulated that hyaluronidase facilitates tumour spread [27], and inhibitors of testicular hyaluronidase found in serum have been proposed to act as tumour growth inhibitors [12]. Anti-inflammatory drugs have been tested using testicular enzyme as a model for hyaluronidase produced in inflammed joints [10].

Such studies would now appear to have been made on a false assumption. This report extends and confirms the early suggestions [28] that serum (and now synovial fluid) hyaluronidase is a distinctly different enzyme from that of testicular origin. The experiments with drugs and serum inhibitors mentioned above, will have to be repeated using human serum enzyme.

In particular, the dependence of serum and synovial fluid hyaluronidase on free thiol groups is more important for their activity than for testicular enzyme, and may prove useful in designing effective inhibitory agents.

If hyaluronidase has a significant degradative role in rheumatoid arthritis and tumour spread mechanisms, then these drugs could possibly be of value in the treatment of such conditions.

Acknowledgements—Synovial fluids used in this study were kindly provided by Drs. R. G. Robinson, D. G. Spencer and J. Webb of the Sutton Rheumatology Laboratories, The Royal North Shore Hospital of Sydney. The support of the National Health and Medical Research Council is gratefully acknowledged.

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