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Characterisation of Norway lobster (*Nephrops norvegicus*) hyaluronidase and comparison with sheep and bovine testicular hyaluronidase

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Abstract

The enzyme used in this study was a partially purified sample of hyaluronidase, extracted and purified from *Nephrops norvegicus* (scampi) hepatopancreas, by acetone fractionation followed by ion-exchange chromatography on Amberlite®IRA 420 and gel filtration on Sephacryl®S-200-HR. The optimum pH varied according to the buffer system, with highest activity being recorded at pH 5.4 in 50 mM sodium-acetate buffer. The enzyme also required NaG at a concentration of 120 mM in the final incubation mixture for optimum activity. Its molecular weight, estimated by gel filtration was 320 kDa and its Km value was 0.42 mg/ml using sodium-hyaluronate from bovine trachea as substrate. The enzyme demonstrated specificity for hyaluronic acid as substrate and showed no activity towards closely related sulphated polysaccharides, chondroitin sulphate A, B or C. On the other hand, the sulphated polysaccharides were found to inhibit scampi hyaluronidase to varying degrees. All enzyme activity was lost on freezing the purified extract to 60°C but addition of dimethyl-sulfoxide (1%) or bovine serum albumin prior to freezing prevented this loss. Scampi hyaluronidase was characterised and compared to commercially available sheep and bovine hyaluronidase. Its specific activity was nearly twice as high as that of the commercial samples. However, scampi hyaluronidase was found to be more sensitive to inhibition by a range of substances. Bovine and sheep hyaluronidase were, however, inhibited to a greater extent than scampi hyaluronidase, by human serum proteins.

Keywords: Hyaluronidase; *Nephrops norvegicus*; Scampi; Characterisation; Properties

1. Introduction

Hyaluronidase (also known as hyaluronoglucosidase, Duran—Reynolds spreading factor and mucinase) is a group of endo-mucopolysaccharidases that depolymerise acid mucopolysaccharides such as hyaluronic acid from connective tissue and chondroitin-sulphate found in cartilage. The pharmaceutical and food industry applications of hyaluronidase were noted by Krishnapillai, Taylor, Morris, and Quantick (in press). Hyaluronic acid, irrespective of its source has been shown to be an unbranched polymer consisting of repeating disaccharide units of (→3) 2-acetamido-2-deoxy-β-D glucose (*N*-acetyl D-glucosamine) and β(1→4) D-glucuronic acid (or D-glucuronate) β(l→3) in a linear, flexible chain (Meyer, 1958). Two major forms of hyaluronidase have been identified depending on the bond hydrolysed in the heteropolysaccharide, hyaluronic acid. One form known as hyaluronoglucosaminidase, hexosaminidase, β-endohexosaminidase and hyaluronoglucosidase causes random hydrolysis of the β(1→4) linkages between *N*-acetyl D-glucosamine and D-glucuronic acid in hyaluronate and it therefore, has the systematic name 4-glycanohydrolase (EC 3.2.1.35). This enzyme also hydrolyses 1,4 β-glycosidic linkages between *N*-acetyl glucosamine or *N*-acetyl galactosamine sulphate and glucuronic acid in chondroitin 6sulphate, chondroitin 4-sulphate and dermatan. The second form of hyaluronidase, also known as hyaluronoglucuronidase and β-endoglucuronidase, causes the random hydrolysis of β(l→3) linkages between D-glucuronate and *N*-acetyl D-glucosamline units in hyaluronate and it therefore, has been given the systematic name 3glycanohydrolase (EC 3.2.1.36).

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Hyaluronidase was first isolated from micro-organisms (Meyer, Hoffman, & Linker, 1960). Subsequently, its presence has been reported in various animal tissues sources (Kiishnapillai, in press). Since hyaluronidase plays a vital role in fertilisation of the mammalian egg, the testis is reportedly the richest mammalian source; consequently commercial hyaluronidase is obtained from bovine and/or sheep testes. Recently, scampi hepatopancreas has also been shown to be a rich source and its extraction and purification by acetone fractionation, ion-exchange chromatography on Amberlite™ IRA 420 (Cl^-) and subsequent gel filtration on Sephadex™ G-200 or Sephacryl™ S-200-HR has been reported (Krishnapillai et al. in press). This paper details the further characterisation of hyaluronidase extracted from scampi hepatopancreas.

2. Materials And Methods

Sodium hyaluronate from bovine trachea and all other chemicals used for monitoring enzyme activity, unless otherwise stated, were obtained from Sigma® Chemical Company. A highly purified hyaluronidase preparation obtained from scampi (*Nephrops norvegicus*) waste by acetone fractionation, ion-exchange chromatography on Amberlite® IRA 420 and finally on Sephacryl® S-200-HR gel filtration media by methods described previously (Krishnapillai et al., in press) was used for characterisation studies unless otherwise stated. Native-PAGE with PhastGel®, gradient 10—15 using PhastGel native buffer strips with protein detection by Coomassie staining had indicated contamination by two other proteins (Krishnapillai et al.).

2.1. Enzyme assay

Hyaluronidase activity was measured as previously described (Krishnapillai et al., in press).

2.2. Effect of pH

The effect of pH in various buffer systems was studied using 50 mM buffers (as listed below) containing 0.15 M NaCl, and a 10 min incubation time:

- Sodium-phosphate buffer pH 5.0 to 8.0
- Sodium citrate buffer pH 3.0 to 6.0
- Tris-HO buffer pH 4.0 to 8.0
- Sodium acetate buffer pH 3.0 to 6.4

2.3. Temperature–activity profile

The temperature–activity profile of the enzyme was studied from 5 to 70°C. Two series of incubations were carried out for 10 and 20 min, respectively.

2.4. Effect of enzyme enhancers/inhibitors

To 100 µl of the enzyme sample in 50 mM sodium-acetate buffer, pH 5.4, was added 200 µl of the enhancer/inhibitor, dissolved in distilled water, at the required concentration. After being left at 25°C for 30 min to attain equilibrium, enzyme activity was initiated by the addition of 200 µl sodium hyaluronate (3 mg/ml) in 100 mM. sodium–acetate buffer at pH 5.4, containing 0.3 M NaCl (final concentration in incubation mixture: 120 mM). Each sample was incubated for 20 min, with a standard being run simultaneously under similar conditions but without inhibitor or enhancer in the buffer. The enzyme activities were then calculated as a percentage of the standard.

2.5. Molecular weight determinations

Sephacryl® S-200-HR gel filtration medium (Pharmacia® LKB Biotechnology obtained from Sigma® Chemical Company) was packed into a chromatographic glass column (2.6 × 65 cm, i.d. × 1, supplied by Pharmacia® LKB Biotechnology) to obtain a gel bed height of 49 cm. The column was equilibrated and run with 10 mM sodium–acetate buffer, pH 5.4, containing 5% glycerol and 0.15 M NaCl.

The void volume (V_o) was estimated using blue dextran. The column was calibrated using Gel Filtration Molecular Weight Markers (MW-GF-1000, Sigma® Chemical Company). Individual protein solutions (2 ml), including highly purified hyaluronidase, were applied to the column and their elution volumes (V_e) estimated by monitoring absorbance at 280 nm. The molecular weight of scampi hyaluronidase was estimated from a standard plot of log M.wt. against the ratio V_o/V_e of the gel filtration markers.

2.6. Comparative studies with other commercially available hyaluronidase

The pH profile, NaCl requirement, Km and the effect of various enhancerslinhibitors on activity of two commercially available, highly purified 4-glycanohydrolase preparations from sheep testes (lyophilised powder, Sigma® catalogue no. H-6254) and bovine testes (chromatographically purified, dialysed and lyophilised powder, Sigma® catalogue no. H-3631) were compared with those of purified scampi hyaluronidase.

3. Results And Discussion

All results presented in this study are the average values obtained from triplicate experiments, unless otherwise stated.

The optimum pH for activity of hyaluronidase was dependent on the buffer system used (Fig. 1). This

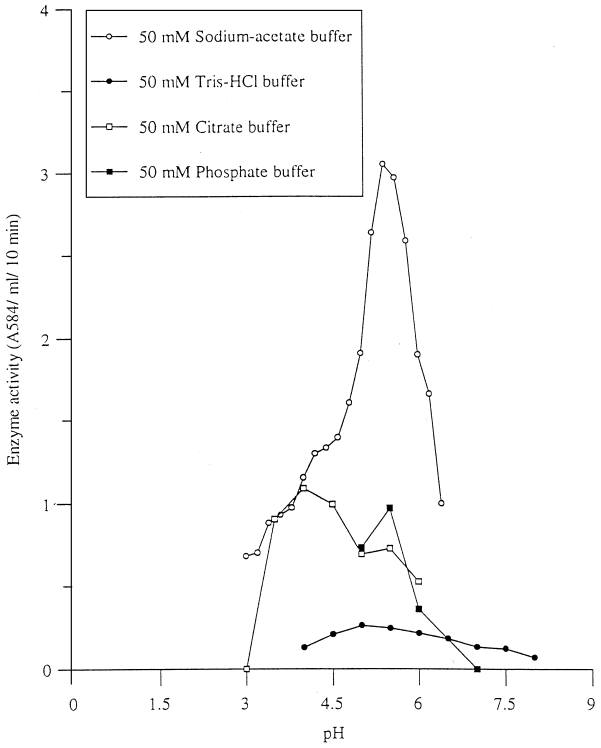

Fig. 1. Effect of pH in various buffer systems (50 mM) on the activity profile of purified *N. norvegicus* hyaluronidase.

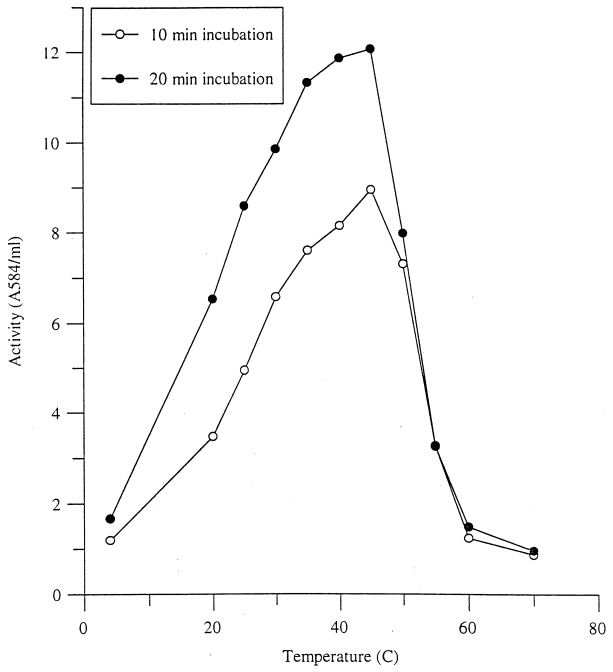

Fig. 2. Temperature activity profile of scampi hyaluronidase preparation purified by acetone fractionation, anion-exchange and gel filtration chromatography.

phenomenon was also reported for rat and guinea pig kidney hyaluronidase, the optima for which were pH 3.8 in 100 mM sodium—acetate and pH 4.2 in 50 mM citrate-phosphate (Bollet, Bonner, & Nance, 1963).

The highest activity for scampi hyaluronidase was observed in 50 mM sodium-acetate buffer with a sharp optimum activity at pH 5.4 (Fig. 1). Hyaluronidases from most animal sources have pH optima in the acidic range, between 3.8 and 5, whereas most bacterial sources have optimum activity in the near neutral range of pH 6–7.

A time-course of enzyme activity studied with a purified preparation of scampi hyaluronidase at a concentration of 0.25 mg/ml (protein estimated by the method of Lowry, Rosebrough, Farr, & Randall, 1951) showed that the amount of product formed from the substrate by the action of hyaluronidase showed a linear increase with time at 25°C, but only up to 25—30 min of incubation.

The temperature–activity profile (Fig. 2) shows the enzyme to have highest activity at 45°C, with a sharp drop in activity above this temperature. This result is rather surprising considering the fact that *N. norvegicus* is a cold water species living in temperate waters. The optimal temperature for hyaluronidase activity from induced *Flavobacterium heparinum* was much lower at 25°C (Aronson & Davidson, 1967), while for constitutive *F. heparinum* and *S. aureus* it was 37°C. The hyaluronidase from leech extremities, which is a β-endoglucuronidase has optimum activity at 38°C (Zeller, 1948). Yang and Srivastava (1975) found that bovine testicular hyaluronidase had optimum activity at 37°C, but at 50°C in the presence of gelatine, it was twice as active as at 37°C. They also reported that bacterial hyaluronidase was almost completely destroyed at 60°C. Meyer (1958), however, found that testicular hyaluronidase was remarkably heat-stable, and had a temperature optimum of 50°C.

The Km value of scampi hyaluronidase was calculated to be 0.42 with hyaluronate as substrate but the enzyme showed no activity towards chondroitinsulphate A, B or C in a 30 min incubation study. Prolonged incubation of the purified enzyme (approximately 12 h) with chitin, resulted in detection of *N*-acetylglucosamine using the method described by Reissig, Strominger, and Leloir (1955).

The effect of various amino acids and metal ions on the activity of scampi hyaluronidase was investigated. The metal ions were studied at 8×10^{-4} M concentration, and mercuric chloride, copper sulphate, ferric chloride and ferrous chloride completely inhibited the enzyme, while silver chloride resulted in 82% inhibition. On the other hand, cobalt chloride and lead nitrate enhanced the enzyme activity by 18%. EDTA also enhanced enzyme activity by up to 24%.

Di and tri-valent metal ions (Fe^{+++}, Cu^{++}, Fe^{++} and Zn^{++}) have been reported to be reversible inhibitors of hyaluronidase, the inhibition being reversed by cysteine or pyrophosphates by chelating the metal ions (Meyer, 1958). Ferric chloride has been used as a

flocculant for hyaluronidase extraction from shrimp processing waste waters (Olsen, Johansen, & Myrnes, 1990). However, ferric ions have been reported to be potent inhibitors of human kidney hyaluronidase. Ferrous ions were also inhibitory but to a much lesser extent (Bollet et al., 1963). Bovine testicular hyaluronidase was not affected by cobalt, copper or zinc ions (Borders & Raftrey, 1968), whereas scampi hydaluronidase was enhanced by cobalt, totally inhibited by copper and partially inhibited by zinc ions. Cysteine caused complete loss of enzyme activity and arginine and aspartic acid inhibited the enzyme activity by 52 and 32%, respectively.

Bovine serum albumin (BSA), at 0.26 mg/ml, enhanced enzyme activity by upto 22% over the control sample. Freezing and thawing purified hyaluronidase samples caused complete loss of enzyme activity; however, addition of BSA to the enzyme sample prior to freezing resulted in complete retention of enzyme activity. Addition of dimethyl-sulfoxide to a final concentration of 1% in the mixture serves the same function.

Using the calibration curve obtained with the gel filtration molecular weight markers MW-GF-1000 (Sigma® Chemical Co.), hyaluronidase from scampi waste was estimated to have a molecular weight of ≈320 kDa. This study was repeated four times with reproducible results.

Table 1 shows the molecular weight of hyaluronidase extracted from various sources. The molecular weight of purified bovine testicular hyaluronidase was found to be 61 kDa as opposed to 126 kDa for the crude enzyme, this difference being attributed to either the association of the crude enzyme with a carrier protein or to the existence of the crude enzyme as a dimer (Borders & Raftrey, 1968).

Table 1
Molecular weight of hyaluronidase from various sources

Source	Molecular weight (Da)	Method employed
Rat liver lysosome	89,000	Ultra centrifugation (sedimentation equilibrium) (Aronson & Davidson, 1967)
Bovine testes	126,000 (crude enzyme); 61,000 (purified enzyme)	Gel filtration on Sephadex® (Borders & Raftrey, 1968)
Bull sperm	62,000	SDS gel electrophoresis (Yang & Srivastava, 1975)
Chick embryo skin and fibroblasts	62,000	Gel filtration on Sephadex® G-200 Orkin & Toole, 1980)
Shrimp processing waste waters	50,000	Not specified (Olsen et al., 1990)
Scampi hepatopancreas	320,000	Gel filtration on Sephacryl® S-200-HR

Native-PAGE of the purified scampi extract on PhastGel®, gradient 10–15 using PhastGel native buffer strips indicated three protein bands within the molecular weight range of 280 to 390 kDa.

Studies on scampi and commercial preparations of bovine and sheep testis hyaluronidase showed differing characteristics (Table 2). All three hyaluronidase preparations had pH optima between 4.4 and 5.4. Sheep hyaluronidase had the highest Km value at 4.2 mg/ml, and scampi hyaluronidase had the lowest Km at 0.42 mg/ml. The lowest Km reported for hyaluronidase is 0.08 mg/ml for rat liver hyaluronidase (Aronson & Davidson, 1967). Bull-sperm hyaluronidase has a Km of 3.7 mg/ml (Lowry et al., 1951) which is similar to that determined for bovine testis (3.2 mg/ml). Scampi hyaluronidase has a sharp optimum NaCl requirement of 120 mM in the final incubation mixture (Fig. 3) and

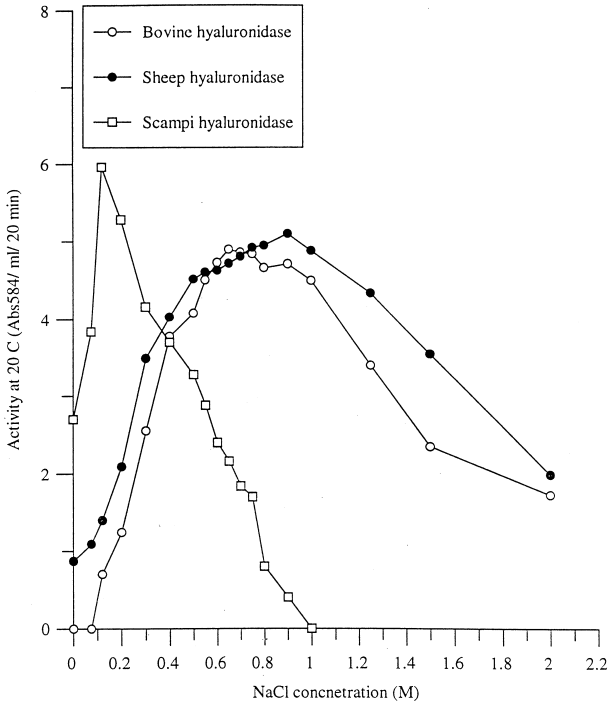

Fig. 3. Effect of NaCl on the activity of hyaluronidase from bovine and sheep testes and *N. norvegicus* hepatopancreas.

Table 2
Comparison of pH optima, NaCl requirement and Km values for hyaluronidase from scampi hepatopancreas, bovine and sheep testes

Source of hyaluronidase	pH optimum[a]	Optimum NaCl concentration (M)[b]	Km value (mg/ml)[c]
Scampi	5.4	0.12	0.42
Bovine testis	4.4	0.65	3.2
Sheep testis	4.8	0.90	4.25

[a] In 50 mM sodium acetate buffer.
[b] In final incubation mixture.
[c] Using hyaluronic acid from bovine trachea as substrate.

concentrations over 1.0 M caused total inhibition. However, both bovine testicular hyaluronidase (b.t.h.) and sheep testicular hyaluronidase (s.t.h.) had a broad plateau of NaCl requirement with optimum activity recorded at 0.65 M for b.t.h. and 0.9 M for s.t.h. in the final incubation mixture. Yang and Srivastava (1975) reported that bull-sperm hyaluronidase had an absolute NaCl requirement for its activity, but the optimum values were not given. They also reported that, in the presence of chondroitin-sulphate B, a potent inhibitor of hyaluronidase, the NaCl requirement in the incubation mixture was higher. However, rat liver hyaluronidase of lysosomal origin did not require NaCl for optimum activity.

Table 3
Comparative activities of hyaluronidase from scampi hepatopancreas, bovine testis and sheep testis

Source	Protein (mg/ml)[a]	A584/ml/20 min at 20°C	A584/mg/20 min at 20°C
Scampi	0.038	1.9	50
Bovine	0.217	5.38	25
Sheep	0.13	4.26	33

[a] Protein estimated by Lowry method (Lowry et al., 1951).

The highly purified hyaluronidases from scampi hepatopancreas, bovine and sheep testes, were incubated with each substrate at their optimum pH and NaCl concentrations for activity. The results obtained from 20 min incubations are shown in Table 3. The specific activity of the highly purified scampi hyaluronidase was found to be 1.5 times higher than sheep hyaluronidase and double that of bovine testicular hyaluronidase. Bull-sperm hyaluronidase has been reported to be 1.5 times more active than the testicular hyaluronidase (Yang & Srivastava, 1975).

Comparative studies on the effect of various enhancers/inhibitors on the hyaluronidase purified from scampi processing waste, bovine and sheep testes were also performed and results obtained are shown in Table 4. Scampi hyaluronidase was the most sensitive of the hyaluronidases, to most of the enhancerlinhibitors

Table 4
The effect of enhancerslinhibitors on the activity of hyaluronidase from scampi hepatopancreas, bovine and sheep testes

Enhancer/inhibitor	Concentration of enhancer/inhibitor (mg/ml)	% Activity remaining		
		Scampi hyaluronidase[a]	Bovine testes hyaluronidase[b]	Sheep testes hyaluronidase[c]
Chondroitin-sulphate A	1.2	0	70.2	43
	0.12	14.7	91.9	94.3
	0.012	56.3	100	105
Chondroitin-sulphate B	1.2	0	93.3	102
	0.12	0	95.5	105
	0.012	10	102	102
Chondroitin-sulphate C	1.2	0	58.9	71.1
	0.12	11	87.6	101.3
	0.012	46.5	98.3	98.7
Heparin (a)[d]	1.2	41.6	99.3	111
	0.12	76	98.8	102
	0.012	95.8	95.5	101
Heparin (b)[e]	1.2	0	90.7	112.9
	0.12	15.7	94.5	106
	0.012	54.1	94.3	104.7
Glycated human serum protein	1.2	98.9	99.3	105
	0.12	100.2	99	99.7
	0.012	99.8	100.2	100.7
Frozen human serum protein	0.4	66.3	69.9	64.5
	0.2	93.8	76.6	83.6
Cysteine	1.2	0	0	0
	0.12	0	0	0
	0.012	0	0	0

[a] Purified by anion-exchange and gel filtration.
[b] Chromatographically purified, dialysed and lyophilised (sigma product no. H-3631).
[c] Lyophilised powder (sigma product no. H-6254).
[d] Heparin (a): molecular weight 6000.
[e] Heparin (b): molecular weight not specified.

studied. Of the polysaccharides investigated, chondroitin-sulphate B (or dermatan sulphate) was the most potent inhibitor of scampi hyaluronidase, causing up to 90% inhibition at low concentration (0.012 mg/ml), whilst it had little or no effect at 1.2 mg/ml on s.t.h. or b.t.h. At 1.2 mg/ml concentration, chondroitin-sulphate A (or chondroitin-4-sulphate) caused nearly 60% inhibition of s.t.h., 30% of b.t.h. and complete inhibition of scampi hyaluronidase; even at 0.12 mg/ml the latter was 85% inhibited. Similar results were obtained for chondroitin-sulphate C although b.t.h. was more inhibited in this case than s.t.h. Literature on the enhancers and inhibitors of hyaluronidase from various sources is vast and contradictory in many cases. It has been reported that, although lysosornal hyaluronidase is strongly inhibited by dermatan sulphate (chondroitin-sulphate B), it can act on chondroitin sulphate A and C (Vaes, 1973). However, it does not degrade heparan sulphate or keratan sulphate. The only endoglycosidase identified in mammalian tissues, degrades hyaluronic acid and both isomers of chondroitin sulphate, but degradation of chondroitin sulphates, however, is about 15 times slower than the rate of degradation of hyaluronic acid (Muir, 1973). Testicular hyaluronidase degrades chondroitin sulphate A and C (Meyer, 1958). Lysosomal hyaluronidase from rat liver has been reported to be inhibited by sulphated polysaccharides, most probably through electrostatic interaction (Aronson & Davidson, 1967).

Heparin of low molecular weight (Mw 6000) caused 48% inhibition at 1.2 mg/ml concentration but little or no inhibition of b.t.h. and, at 1.2 mg/ml concentration, it even enhanced the activity of s.t.h. However, it has been reported that heparin and mucin are not substrates of, but competitive inhibitors of testicular hyaluronidase (Meyer et al., 1960).

Frozen and thawed human serum proteins were found to inhibit hyaluronidase activity from all three sources. However, at low concentrations of serum, the inhibitory effect was least for scampi hyaluronidase and highest for b.t.h. Earlier studies have shown that human serum contains both specific (antibodies) and non-specific inhibitors of hyaluronidase (Mann, 1949; Meyer, 1958). The serum inhibitor is thought to be a protein with a molecular weight of about 100,000 which belongs to the class of mucoproteins but is not identical with orosomucoid (Meyer). These non-specific inhibitors have not been completely identified. Finally, cysteine totally inhibited hyaluronidase from all three sources.

4. CONCLUSIONS

The purified hyaluronidase preparation obtained from scampi waste was found to specifically act on hyaluronic acid and had no activity towards closely related sulphated polysaccharides, chondroitin sulphate A, B or C. The enzyme had optimum activity in 50 mM sodium—acetate buffer at pH 5.4 containing 120 mM NaCl. Highest activity was observed at 45°C in a 10 min incubation study. The Michaelis constant for the enzyme was calculated to be 0.42 mg/ml. The salts of ferric chloride, ferrous chloride, copper sulphate and mercuric chloride at 8×10^{-4} M concentration completely inhibited the enzyme. Cysteine at 0.012 mg/ml also completely inhibited the enzyme. Cobalt chloride, magnesium chloride, lead nitrate and EDTA at 8×10^{-4} M concentration enhanced enzyme activity. The enzyme activity was completely lost after freezing to −60°C. However, addition of bovine serum albumin at a concentration of 0.26 mg/ml resulted not only in complete retention of enzyme activity in the frozen sample, but also enhanced the activity by up to 24% in the unfrozen sample. Also, when the sample was frozen in the presence of 1% dimethyl-sulfoxide (DMSO), there was complete retention of enzyme activity.

Two highly purified commercially available hyaluronidase preparations from bovine and sheep testis were characterised with regard to pH optimum, NaCl requirement and Km values. A comparison of the activity of the purified hyaluronidase showed the specific activity of scampi hyaluronidase to be 1.5 to 2 times as high as the cornmercially available samples. The Km value of scampi hyaluronidase was lowest (0.42 mg/ml) followed by bovine (3.2 mg/ml) and sheep (4.25 mg/ml) hyaluronidase. An extensive comparative study of scampi hyaluronidase and sheep and bovine hyaluronidase was performed with various enhancer-slinhibitors. Results showed scampi hyaluronidase to be more sensitive to most of the inhibitors studied, except frozen human serum proteins which inhibited bovine and sheep hyaluronidase to a greater extent.

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