ELSEVIER

Food Chemistry 65 (1999) 359–365

Food Chemistry

Extraction and purification of hyaluronoglucosidase (EC 3.2.1.35) from Norway lobster (*Nephrops norvegicus*)

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Received 13 August 1998; received in revised form; accepted 28 September 1998

Abstract

The waste of Norway lobster (*Nephrops norvegicus* also known as Dublin Bay prawn and scampi) constitutes 75–80% of the whole animal by weight. This includes the hepatopancreas which is a good source of hyaluronoglucosidase (EC 3.2.1.35), commonly referred to as hyaluronidase. This enzyme was partially purified by acetone fractionation, ion-exchange column chromatography on a Type-I polystyrene-based anion-exchange resin, Amberlite™ IRA 420 and subsequently by gel filtration on Sephadex™ G-200. The anion-exchange step of the purification was optimised by including a wash with 10 mM sodium acetate buffer containing 12.5 mM NaCl after loading the column with the enzyme preparation. This was followed by gradient elution with 12.5–500 mM NaCl in 500 ml of the same buffer. The gel filtration step was optimised using Sephacryl™ S-200-HR gel filtration medium. As a result of these modifications to the purification process a 763-fold purification was achieved, with 32% of the enzyme from the original crude extract in 0.25 M sucrose solution being recovered. A loss of 41% of the enzyme activity was recorded during acetone fractionation and 34% during gel filtration. However, recovery from the anion-exchange step using Amberlite™ IRA 420 was as high as 81%. A sample of the purified extract was subjected to native polyacrylamide gel electrophoresis with PhastGel™ Gradient 10-15 using PhastGel Native Buffer Strips which indicated the presence of three proteins.

Keywords: Hyaluronidase; Hyaluronoglucosidase; *Nephrops norvegicus*; Ion-exchange chromatography; Purification; Isolation; Extraction

1. Introduction

The total annual landing of *Nephrops norvegicus* for 1994 in the UK was estimated to be 30,036 tonnes (FAO, 1996) of which almost 75–80% was waste. Several possible ways of utilising crustacean waste have been described, with chitin being the most widely researched component (Baxter, 1990; Brezeski, 1987; Ge, Bai, & Zhang, 1996; Meyers, 1986; Nair, Madhavan, & Gopakumar, 1986). The cephalothorax contains high levels of digestive enzymes (Cann, 1973; Cobb, 1977). Although the recovery of alkaline phosphatase, β-*N*-acety1glucosaminidase, hyaluronidase and chitinase from shrimp processing waste waters of the Northern shrimp (*Pandalus borealis*) processing plants in Norway have been investigated (Olsen, Johansen, & Myrnes, 1990), no attempt has hitherto been made to extract hyaluronidase from solid scampi waste.

Hyaluronidase is an endo-enzyme catalysing the depolymerisation of hyaluronic acid into short chain oligosaccharides. This enzyme has therapeutic applications, as for example, in the cardiovascular field, for salvaging ischaemic tissues following myocardial infarction (Cairns, Holder, Tanser, & Missirilis, 1982; May, Furberg, Eberlein, & Barbara, 1983; Saltissi, Robinson, Coltart, Webb-Peplope, & Croft, 1982; Sanders, 1988). It also has a range of other medical applications (Integral Healthcare, 1993), diagnostic applications, and a minor application in the food industry as a meat tenderiser (Stekol'Nikov, Shilov, Sevastyanov, Belousov, & Mamonov, 1980; Wu, Dutson, & Carpenter, 1981).

Since hyaluronidase is produced in the mammalian testes and plays a role in fertilisation of the mammalian egg (McRorie & Williams, 1974), the enzyme is commercially extracted from mature bovine testes (Borders & Raferty, 1968). Other natural sources of this enzyme include bull sperm (Yang & Srivastava, 1975), leech extremities (Yuki & Fishman, 1962), various mammalian

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0308-8146/99/$—see front matter
PII: S0308-8146(98)00233-7

tissues (Bollet, Bonner, & Nance, 1963), rat liver (Aronson & Davidson, 1967), bacteria (Linker, 1966); human placenta (Mima & Yamada, 1974), chick fibroblasts (Orkin & Toole, 1980) and *Euphausia superba* (Karlstam, 1987). This paper describes the extraction and purification of hyaluronidase (4-glycanohydrolase: EC 3.2.1.35) from the cephalothorax of *N. norvegicus*.

2. Materials and methods

The raw material was whole frozen Norway lobster (*N. norvegicus*) from F.F. Amba®, Denmark. These were hand-shelled and the solid head waste used for the study. Before use, all appendages, outer shell and adhering flesh were removed from the head and the trimmed hepatopancreas so obtained was used as the starting material for the extraction of the enzyme.

Sodium hyaluronate from bovine trachea and other chemicals used for monitoring enzyme activity were obtained from Sigma® Chemical Company. DEAE-cellulose, DEAE Sephacel™, DEAE Sepharose™ CL-6B, Sephadex™ G-200 and Sephacryl™ S-200-HR are products of Pharmacia LKB Biotechnology®, but were obtained through Sigma® Chemical Co. AmberliteTm IRA 420 (Cl−) a product of Rohm and Haas® Co was obtained from BDH® Chemical Co.

2.1. Assay for hyaluronidase activity

Enzyme activity was measured by a modification of the methods described by Linker, Meyer, and Hoffman (1960) and Reissig, Strominger, and Leloir (1955). For a normal assay, 400 μl of the substrate, hyaluronate solution (1.5 mg/ml sodium hyaluronate), in 50 mM sodium-acetate buffer, pH 5.4 containing 150 mM NaCl was incubated with 100 μl of enzyme sample at 25°C for 20 min, unless otherwise stated. Following incubation, 100 μl of 800 mM potassium tetra-borate solution was added, mixed and the test tube heated for 3 min in a boiling water bath and then cooled immediately in cold water. To this was added 3 ml p-dimethylamino-benzaldehyde reagent (10% p-DMAB in glacial acetic acid containing 12.5% HCl). The solution was mixed, incubated for 20 min at 37°C in a water bath and cooled in cold water. The absorbance was measured at 584 nm in a 1 cm glass cell. If the solutions were turbid they were centrifuged to clear before reading. Enzyme activity was expressed as increase in absorbance at 584 nm after a 20 min incubation at 25°C.

2.2. Protein measurements

Protein concentrations were determined by the Protein Assay Procedure No. P 5656 provided by the Sigma® Chemical Company (1989), using crystalline bovine serum albumin as the standard. The method is essentially the Lowry method as modified by Peterson (Lowry, Rosebrough, Farr, & Randall, 1951; Peterson, 1979).

For protein estimation during column chromatography studies, the absorbance (E) was measured at 280 and 260 nm. The E_{280}/E_{260} ratio was calculated and the appropriate correction factor given by Warburg and Christian as cited by Kresze (1983) was applied to obtain the protein concentration from the formula:

$$\text{Protein concentration (mg/ml)} = \text{Factor} \times E_{280}.$$

2.3. Extraction and partial purification of hyaluronidase

A modification of the extraction method of Aronson and Davidson (1967) was used. About 40 g of frozen scampi hepatopancreas was homogenised with an equal volume of cold 0.25 M sucrose at 4°C using a Ystral Stick homogeniser. The nuclear fraction, broken cells and other debris were removed by centrifugation at 1000 ***g*** (r_{av} 10.9 cm) for 30 min in a MSE 18 centrifuge operating at 4°C. The supernatant fluid was diluted with 0.25 M sucrose at 4°C to yield 100 ml of extract solution. This supernatant, termed the ''crude extract'', was found to contain the enzyme hyaluronidase.

Partial purification of the enzyme from the crude extract was carried out by fractional precipitation using ammonium sulphate, polyethylene glycol 6000 and cold acetone. Acetone fractionation without pH adjustments was found to be the most effective, and the 1:0.7–0.9 (extract to acetone) fraction recovered high enzyme activity.

2.4. Chromatographic techniques

The acetone fractionated enzyme preparation was dissolved in 30 ml cold 0.25 M sucrose and left overnight at 4°C. This was then centrifuged at 1000 ***g*** (r_{av} 10.9 cm) for 15 min to remove insoluble fractions and the clear supernatant used for chromatographic studies.

Preliminary experiments conducted with DEAE-cellulose and CM-cellulose to establish buffer pH and ionic strength in 10 mM sodium–acetate buffer for the ion-exchange chromatographic process showed that the enzyme is preferentially bound to the anion-exchanger, DEAE-cellulose, at pH 5.0 and 5.5, while it is not bound to the CM-cellulose in the pH range 4.0–6.0 with the same buffer. Also it was determined from preliminary experiments that the enzyme is released from the DEAE-cellulose resin at a NaCl concentration between 0.15 and 0.25 M. The first chromatographic run was, therefore, made using a 48 cm bed height of

DEAE-cellulose packed into a chromatographic glass column 2.6 × 65 cm (i.d.×1) with a continuous NaCl gradient from 0 to 0.5 M, in 500 ml of sodium-acetate buffer pH 5.0, at 4°C, at a flow rate of 0.7 ml per min. Fractions (7 ml/fraction) were collected and assayed for hyaluronidase activity and protein content. Separate chromatographic runs were also performed with DEAE Sepharose™ CL-6B, DEAE Sephacel™, and Amberlite™ IRA 420 (Cl^-) anion-exchange resins, under the conditions described for DEAE-cellulose.

2.5. Gel filtration studies

A suitable volume of the concentrated fractions obtained from anion-exchange column chromatography was subjected to gel filtration chromatography. About 30 g of Sephadex™ G-200 gel was prepared by allowing the resin to hydrate and swell in water at 80°C for 8 h, cool overnight to the operating temperature of 4°C and it was degassed under vacuum before packing, to ensure removal of all air bubbles from the gel.

A chromatographic column of 2.6 × 65 cm (i.d×1, supplied by Pharmacia™ LKB Biotechnology) was packed using a 75% gel slurry to obtain a final bed height of 49 cm. Packing and elution was performed using 10 mM sodium–acetate buffer, pH 5.4, with 150 mM NaCl and 5% glycerol. The 0.15 M NaCl was added to safeguard against possible ionic interactions with the gel matrix, and the 5% glycerol was added to prevent possible non-specific hydrophobic protein–resin interactions during elution. The elution flow rate was 0.25 ml/min at a temperature of 4°C. Blue dextran at 2 mg/ml concentration was used to test even packing of the column. Size exclusion chromatography was also conducted using Sephacryl™ S-200-HR gel filtration medium and the running conditions were optimised.

3. Results and discussion

Hyaluronidase activity was detected only in the hepatopancreas of scampi. Sequential fractionation of animal tissue homogenates in isotonic sucrose at 600, 10,000 and 105,000 g gives pellets enriched in nuclear, mitochondrial and microsomal fractions respectively (Ozols, 1990). Based on this technique, rat liver hyaluronidase was found to be located in the lysosomal fraction (Kresze, 1983). However, when a crude extract of scampi hepatopancreas in 0.25 M sucrose was centrifuged at 12,500 ***g*** (r_{av} 10.7 cm) for 1 h, the precipitate had no hyaluronidase activity. This suggests that scampi hyaluronidase is possibly not of lysosomal origin. Also, the enzyme is not membrane-bound as neither the use of Triton X-100 (1% v/v) nor monoethylene glycol (1% v/v) in the extraction medium improved the extraction of the enzyme.

From the results of a series of acetone fractionation studies, it was decided that the 1:0.7–0.9 (crude extract: acetone) fraction was most suitable for further purification studies as it yielded 53% recovery with 9-fold purification. This crude enzyme preparation was termed the "acetone fraction".

3.1. Ion-exchange chromatography

Initial ion-exchange chromatographic runs performed on a DEAE-cellulose column showed extremely poor recovery of the enzyme from the column. A maximum 323-fold purification could be obtained in a fraction but recovery in this fraction was only 2.5% of the total chromatographed enzyme. Similar chromatographic experiments with DEAE Sepharose™ CL-6B and DEAE Sephacel™ also yielded poor recoveries of the enzyme from the column. As a result of the poor enzyme recovery with the previous media, the use of Amberlite™ IRA 420 (Cl^-) (a type-I strong anion-exchanger of cross linked polystyrene) as the anion-exchange resin was investigated. The column was first eluted using 10 mM Na–acetate buffer, pH 5.0 with a continuous NaCl gradient from 0.1 to 0.5 M.

This was then modified by using a continuous NaCl gradient from 0 to 0.5 M which resulted in a similar elution but with better separation. Fractions showing high enzyme activity (fractions 19–24) were combined and concentrated by ultrafiltration. Eighty percent of the enzyme activity was recovered from this stage, resulting in an overall 137-fold purification. This suggests that the loss of much enzyme activity on the cellulose and agarose based ion-exchange chromatographic media was due to the enzyme being adsorbed onto the cellulose and/or agarose resin material, possibly due to its active site binding to the gel matrix, which has repeating hexose units with β(1→4) linkages.

Previous studies have also reported difficulties associated with purification of this enzyme on polysaccharide based ion-exchange media. For example, purification of testicular hyaluronidase required the column to be eluted with 5.5 litres of buffer before any enzyme could be eluted from the column (Borders & Raferty, 1968). A direct Sephadex™ purification step before ion-exchange yielded only 1.5-fold purification compared to the starting material. Yuki and Fishman (1962) achieved only 8.3% recovery of hyaluronidase from leech extremities by anion-exchange chromatography on DEAE-cellulose, and further attempts at purification on DEAE-cellulose or calcium–phosphate gel were unsuccessful.

Retention of charged molecules on an ion-exchange column is related to the volume of the column, the molarity of the eluant and its gradient. Short steep gradients are known to give faster separations and sharper peaks but retention between peaks will be reduced

(Ozols, 1990). Thus, to optimise the purification of hyaluronidase by ion-exchange chromatography, 11.5 ml (from a 25 ml fraction) of the 1:0.65–0.9 (crude extract:acetone) fraction, of a hyaluronidase preparation obtained from 40 g of scampi waste, was chromatographed on the Amberlite™ column and eluted using a modified two stage ionic gradient elution.

Stage 1 used a linear NaCl gradient from 0 to 0.025 M in 500 ml of 10 mM sodium–acetate buffer at pH 5.0, and stage 2 a linear NaCl gradient from 0.025 to 0.75 M in 500 ml of 10 mM sodium-acetate buffer at pH 5.0. A total buffer volume of 370 ml was allowed to flow through the column during stage 1 at a rate of 0.77 ml/min after which stage 2 was begun with the same flow rate. Fractions eluting from the column were collected at a rate of 15.4 ml/fraction during stage 1 and at a rate of 7.7 ml/fraction during stage 2.

The protein fraction containing enzyme activity was now well separated from the various protein fractions without any enzyme activity. The fractions containing enzyme activity were pooled and concentrated by ultrafiltration as before. The enzyme preparation obtained showed 71.5% recovery of the chromatographed enzyme activity with a 125-fold purification.

However, during stage 1 of the elution, two protein peaks emerged within a NaCl concentration of <0.013 M prior to any enzyme activity. Thus a NaCl concentration of 0.0125 M in the starting buffer would prevent these two protein peaks from being adsorbed onto the column. Subsequently, there would theoretically be more available counter charges left on the column material for exchange with the charged protein fraction of interest. It was considered therefore, that it might be possible to increase the initial concentration of crude extract that could be loaded onto the column, without any of it being lost in the wash phase.

These two possibilities were tested by using the 1:0.65–0.9 (crude extract:acetone, v/v) fraction dissolved in 60 ml of 10 mM sodium acetate buffer, pH 5.4 containing 0.0125 M NaCl, obtained from 80 g scampi waste. A 55 ml volume of this solution was applied onto the Amberlite™ IRA 420 column and washed with the same buffer (10 mM Na–acetate buffer, pH 5.4 containing 0.0125 M NaCl), until the UV recording suggested little or no protein in the eluate. The column was then eluted using a linear NaCl gradient from 0.0125–0.5 M in 500 ml of 10 mM sodium–acetate buffer, pH 5.4 (the pH of the eluting buffer was modified to 5.4, since at this stage characterisation studies had confirmed optimum activity for the enzyme to be at this pH).

The experimental results obtained from this run are shown in Fig. 1. No enzyme activity was detected in the wash phase and a marked improvement in purification was achieved under these conditions of elution. The maximum purification achieved was 405 times the original extract (fraction no. 18). Fractions showing high enzyme activity (fractions 17–23) were pooled and

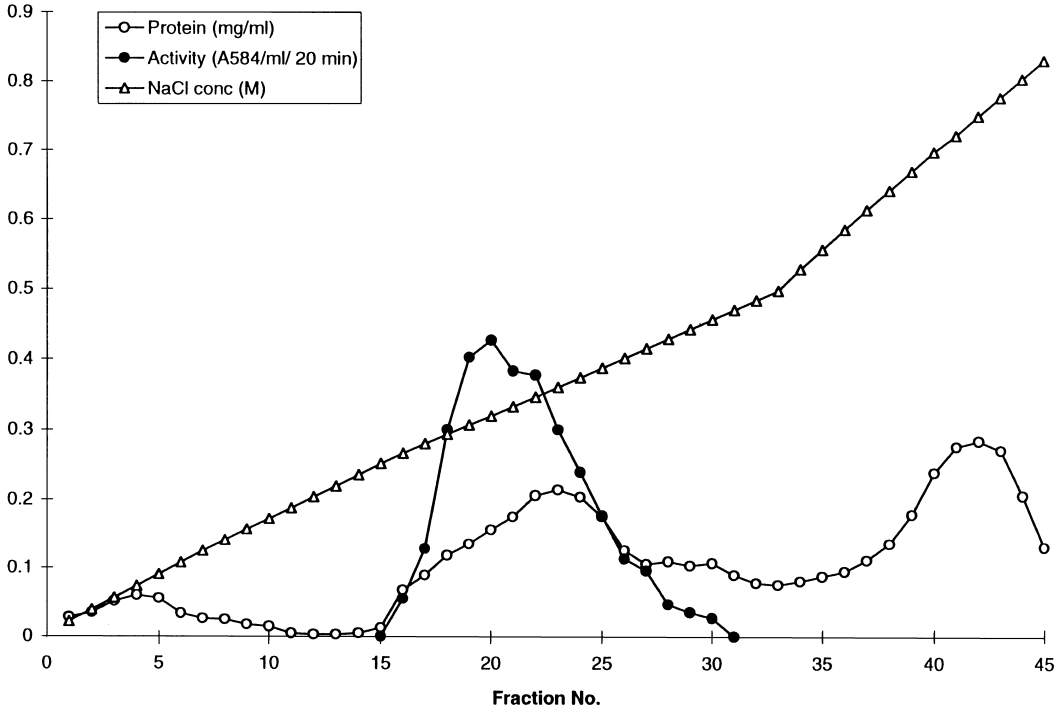

Fig. 1. Results of two stage ion-exchange chromatography of hyaluronidase obtained by acetone fractionation of scampi extract on Amberlite™ IRA. Column: Amberlite™ IRA 420 (2.6 × 48 cm), sample: 10 ml of 0.7–0.9 acetone fraction of scampi extract (30 ml), buffer: 10 mM sodium acetate pH 5.4, two stage elution (see text), flow rate: 1.0 ml/min (15 ml/fraction).

concentrated by ultrafiltration as before. This concentrated sample referred to as IEC (Table 1) showed a 257-fold purification and an 81% recovery of the actual enzyme sample chromatographed on the column. These loading and eluting conditions were tested over five times on the same column with reproducible results.

3.2. Gel filtration with Sephadex™ G-200

With Sephadex™ G-200, as the gel filtration medium, the maximum flow rate obtained was only 0.21 ml/min, resulting in the gel filtration run-time being in the order of about 24 h. Any attempt to increase flow-rates caused the gel material itself to be forced out through the supporting netting of the lower end-piece. Excess pressure resulting from increased pump speeds also caused collapse of the gel matrix.

To reduce the time required for a gel filtration run, Sephacryl™ S-200-HR was used in place of Sephadex™ G-200 medium. This is a cross-linked acrylamide gel described by Pharmacia® as a fast-flow, high-resolution matrix.

Table 1
Summary of results of optimised purification of hyaluronidase by ion-exchange chromatography on Amberlite™ IRA 420 followed by gel filtration on Sephacryl™ S-200-HR

Sample	Volume (ml)	Protein (mg/ml)	A584/ml/ 20 min	A584/mg/ 20 min	Recovery (% chromatographed)	Purif. fold	Recovery (% of original)
Crude extract	200	47.1[a]	3.47	0.074	–	1	100
		8.05[b]		(0.43)[c]		(1)[c]	
0.65-0.9 acetone	60	5.04[a]	6.84	1.36	–	18	59
fraction		2.16[b]		(3.17)[c]		(7)[c]	
55 ml of the acetone fraction purified on Amberlite™ IRA420.							
Ion-exchange	34	0.472[a]	8.94	18.9	81	257	48
concentrate (IEC)		0.533[b]		(16.77)[c]		(39)[c]	
Run 1:12 ml of IEC purified on Sephacryl™ S-200-HR.							
Gel filtered	11.6	0.108[a]	6.1	56.5	66	766	32
sample (SHR)		0.0752[b]		(81.3)[c]		(189)[c]	

[a] Protein values estimated by A280/260 method (Kresze, 1983).
[b] Protein values estimated by Lowry method (Lowry et al., 1951).
[c] Results in parentheses are based on protein values obtained by Lowry method (Lowry et al., 1951).

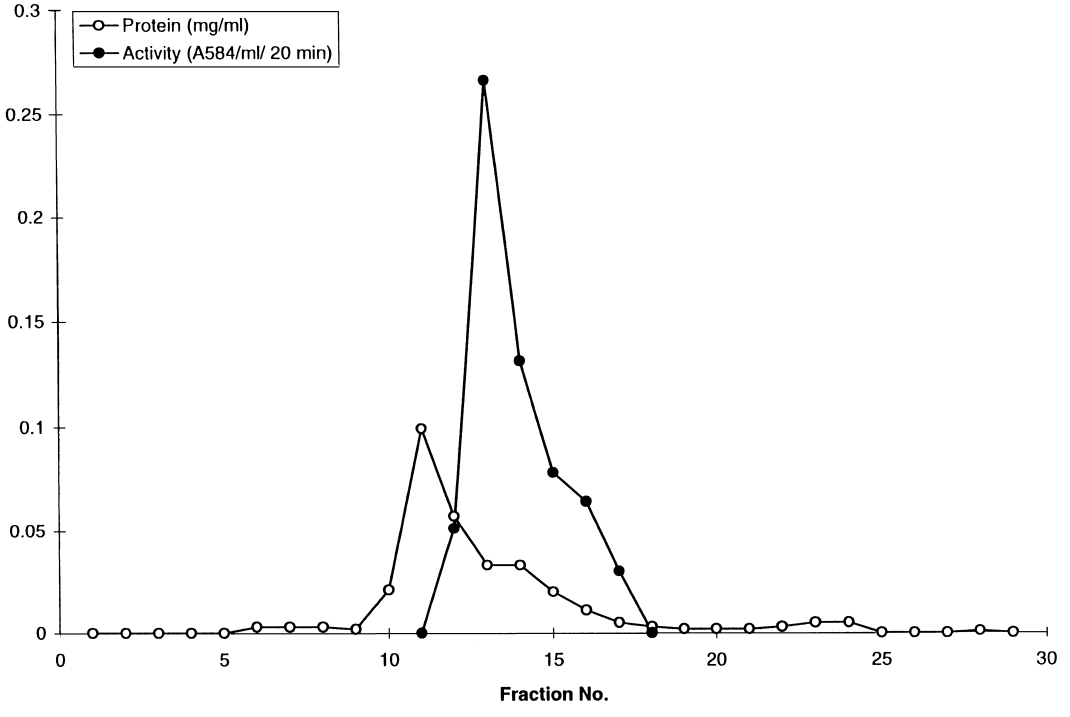

Fig. 2. Results of Sephacryl™ S-200-HR gel filtration chromatography of hyaluronidase preparation IEC. Column: Sephacryl S-200-HR (2.6 × 49 cm), sample: 10 ml of IEC (see text), buffer: 10 mM sodium acetate pH 5.4, flow rate: 1.1 ml/min (11.1 ml/fraction).

Two further runs were made on the Sephacryl™ gel filtration column using the hyaluronidase preparation IEC. During the first run no glycerol was added to the eluting buffer. The results are shown in Fig. 2 and summarised in Table 1 as SHR. Calculations from the concentrated fractions for Run-1 (SHR) shows 760-fold purification with 66% recovery of the enzyme sample that was chromatographed on the column. Run-2 was performed on the same column under similar conditions except that glycerol and NaCl were excluded from the buffer. The results were comparable to those obtained using buffer containing glycerol and NaCl. Thus these components of the eluting buffer are not essential. Fraction no. 13, which represented 33% of the chromatographed enzyme, and had the highest purification (1080 times the original) was used for further enzyme characterisation studies. The concentration of protein in the crude extract, and to a lesser extent in the acetone precipitate, was lower when estimated by the Lowry method (Lowry et al., 1951) than by the UV method. The difference in these protein values could be due to absorbance by phenolic components present in scampi waste (Yan, Taylor, & Hanson, 1989) giving apparently high results when estimated by the UV method.

A summary of the results of optimised purification of hyaluronidase from scampi waste by ion-exchange chromatography on Amberlite™ IRA 420 (IEC) followed by size exclusion chromatography on Sephacryl™ S-200-HR (SHR) are shown in Table 1. All runs under optimised conditions using Amberlite™ IRA420 and Sephacryl™ S-200-HR were repeated several times and found to be reproducible.

The purified enzyme extract (fraction no. 13 from Sephacryl™ S-200-HR, described above) was subjected to native polyacrylamide gel electrophoresis (native-PAGE) with PhastGel™, gradient 10–15 using PhastGel native buffer strips. The protein bands were detected by the optimised methods for detecting proteins in PhastGel gradient media by Coomassie staining (Pharmacia's PhastSystem™ Separation Technique File no. 120 and Development Technique File no. 200). The results indicated the presence of three proteins.

4. Conclusions

Purification of hyaluronidase (EC 3.2.1.35) from scampi hepatopancreas was achieved by acetone fractionation, ion exchange column chromatography on Amberlite™ IRA 420 and subsequently by Sephadex™ G-200 gel filtration. A 457-fold purification of the enzyme was obtained with 19% recovery. The final purification of hyaluronidase from scampi hepatopancreas was optimised on Amberlite™ IRA 420 anion-exchange column using a gradient elution between 12.5 mM to 500 mM NaCl in 500 ml of 10 mM sodium–acetate buffer at pH 5.4. This was followed by gel filtration on Sephacryl™ S-200-HR, the column being eluted with 10 mM sodium–acetate buffer at pH 5.4. Addition of NaCl or glycerol to the eluting buffer was found not to be essential for gel filtration. Under the optimised conditions of chromatography on Amberlite™ IRA 420, followed by Sephacryl™ S-200-HR, a final 763-fold purification and 32% recovery was achieved. A fraction of the highly purified extract, when subjected to native polyacrylamide gel electrophoresis (native-PAGE) with protein detected by Coomassie staining, indicated contamination by two other proteins.

Purification attempts by other methods such as DEAE cellulose, DEAE Sepharose™ CL-6B and DEAE Sephacel™ columns resulted in very low recoveries of the enzyme, possibly due to loss of enzyme by binding to the medium by the active site.

Acknowledgements

The authors wish to thank Rotary International for providing financial support for this research.

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