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An approach for a rapid identification and characterization of hyaluronic acid produced by fermentation of *Streptococcus equi*

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An approach for a rapid identification and characterization of hyaluronic acid (HA) produced by fermentation of *Streptococcus equi* has been developed. ^{125}-I-labelled hyaluronic acid binding protein (HABP) was used for specific identification of HA in fermentation isolates recognized by HPLC chromatography followed by γ-radiation detection. Simultaneous determination of the molecular weight of HA was carried out also. Further confirmation of HA identification was performed by bovine testes hyaluronidase treatment and HPLC analysis of the products. The analysed sample was identified as almost pure HA with molecular weight approximately 0·8–1·2 × 10^6 Da, containing protein impurities (5·1%) and some unidentified low molecular weight products. Our approach can be used generally for a rapid identification and characterization of laboratory or industrially-produced HA during its isolation and purification.

INTRODUCTION

Hyaluronic acid (HA) is a naturally occurring long-chain unbranched high viscosity glycosaminoglycan consisting of a repeating disaccharide unit (Glcβ1 → 3GlcNAc)β1 → 4-linked, which has a broad distribution in vertebrate connective tissue (Laurent, 1970). Its molecular weight varies from 10^4 to 13·0 × 10^6 Da. Over the past 10 years there have been a number of studies reporting potential therapeutic applications of purified sodium hyaluronate.

Indications of pharmacological activity have been demonstrated in the treatment of inflammatory and degenerative joint diseases in both man and animals (Asheim & Lindbald, 1976; Numiki *et al.*, 1982), prevention of postoperative tendon sheath adhesion formation (St. Onge *et al.*, 1980) and facilitation of wound healing (Stenfors, 1987). Additionally, sodium hyaluronate preparations have been used as a replacement of the vitreous fluid during ophthalmic surgery (Miller & Stegmann, 1983) and various forms (gels, tubes, membranes) have been used in animal studies for matrix engineering, the purpose of which is to control and direct tissue regeneration and augmentation (Balazs & Denlinger, 1990). Therefore, increasing interest in the industrial production of HA can be observed (Balazs, 1979; Balazs & Leshchiner, 1987; Hosoya & Kimura, 1987). The main problem in the industrial production of HA is rapid identification and characterization of the HA produced, in order to evaluate its quality during the isolation process.

The aim of this work was a development of an approach useful for a rapid identification and characterization of extracellular hyaluronic acid produced during the fermentation of *Streptococcus equi* into the cell culture medium.

MATERIALS AND METHODS

The sample studied was lyophilized semicrude extract from the fermentation medium of *Streptococcus equi*, Czechoslovak Collection of Microorganisms, CCM 5532, Brno, after alcohol precipitation. This was obtained from Dr L. Velebný, CONTIPRO, Czechoslovakia. Hyaluronic acid (HA) standards (0·8 × 10^6, 1·6 × 10^6 and 4 × 10^6 Da) were purchased from Pharmacia, Sweden. Hyaluronidase (EC 3.2.1.35)

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isolated from bovine testes, with an activity of 237 TRU/mg (declared by the producer) was obtained from SEVAC, Prague, Czechoslovakia. Hyaluronic acid binding protein labelled by ^{125}I (^{125}I-HABP) was isolated from bovine nasal cartilage according to the method of Tengblad (1980) and labelled by the method of Laurent & Tengblad (1980). Other chemicals used were p. a. grade and obtained from LACHEMA, Brno, Czechoslovakia.

High performance gel exclusion liquid chromatography

This was performed on a Beckman System Gold 126 Programmable Solvent Module instrument equipped with a size exclusion column HEMA-BIO-1000 column (8 × 250 mm), 100–3000 kDa (Tessek, Prague, Czechoslovakia) and a 20 µl sample loop. Peak elutions were monitored with a Beckman 166 Programmable Detector Module at 206 nm. Retention times and peak integration were recorded by a Shimazu CR6A Chromatograph Integrator. The HPLC elution buffer was 50 mM sodium phosphate, pH 7·8 and flow 0·5 ml/min. All measurements were performed at room temperature according to Laari & Kotinen (1989).

Degradation of the HA sample by the hyaluronidase

The HA sample was dissolved in acetate buffer, 0·1 M, pH 5·0 to give a concentration of 1 mg/ml and preincubated for 30 min at 37°C. Bovine testicular hyaluronidase (122 TRU/mg HA) was added to the sample and incubated at 37°C for 24 h. The reaction products were identified by HPLC as described above.

Evaluation of the affinity to ^{125}I-HABP

This was performed according to Bonnet *et al.* (1985). Samples (20 µl, 0·01 µg) of ^{125}I-HABP (55 cpm/µl) were chromatographed as described above without and with HA preincubation. Fractions (0·5 ml) were collected and their radioactivity were measured by use of γ counter (TESLA NA 3601, Czechoslovakia).

Ultraviolet spectroscopy

This was performed with the single beam registratory UV-spectrophotometer DU-7 (Beckman Instruments, Germany) in 0·15 NaCl solution a concentration of the sample of 0·5 mg/ml at laboratory temperature.

Protein determination

This was performed according to the method of Sedmak & Grossberg (1977). Bovine serum albumin (SERVA, Germany) was used as the protein standard.

RESULTS AND DISCUSSION

Gel exclusion chromatography coupled with HPLC (HPGPC) instrumentation can be advantageously used as an analytical tool for the main steps of identification and characterization of the HA sample. The method is very reproducible, precise and rapid and allows molecular weight determinations up to 4 million to be done in the presence of considerable amounts of impurities. This technique is a considerable improvement on light-scattering sedimentation equilibrium and viscometry methods for molecular weight determination (Beaty *et al.*, 1985).

The gel permeation profile of the sample is shown in Fig. 1(a). The single symmetric peak of the sample was observed at the retention time of 9·03 min which represents a high molecular weight product with very small amounts of higher molecular weight impurities that appeared on the chromatogram as a tail of increased absorbancy at 206 nm. Low molecular weight substance(s) gave a peak with the retention time of 16·9 min. Comparison of the retention time of the macromolecular peak with HA standards allowed us to determine its approximate molecular weight as about 0·8–1·2 × 10^6 Da. Similar or better results could probably be obtained by using the generally used size exclusion column Toyo Soda, TSK 5000/6000 PW.

Further confirmation of the sample as HA was

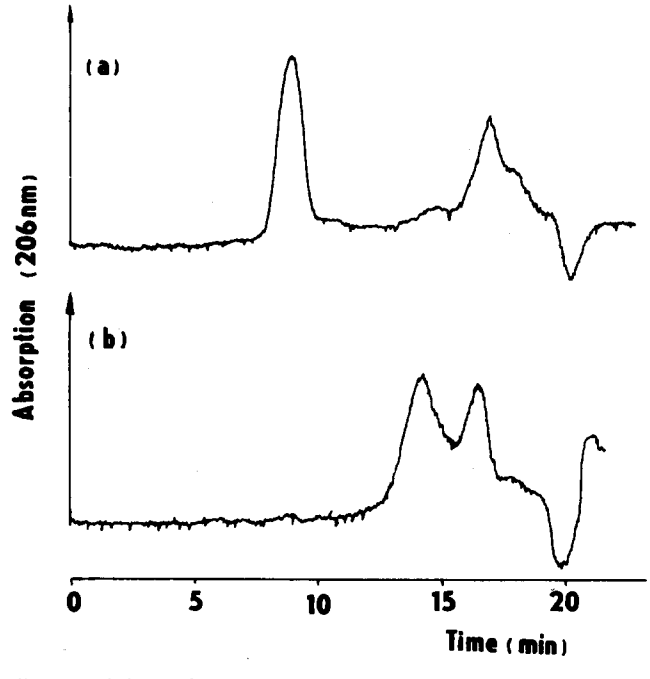

Fig. 1. High performance gel permeation chromatography of the sample from *Streptococcus equi* before (a) and after (b) bovine testes hyaluronidase treatment. Sample (20 µl, 1 mg/ml) was eluted from HEMA-BIO-1000 column (8 × 250) with 50 mM sodium phosphate buffer, pH 7·8, flow 0·5 ml/min at room temperature. Peak elution was monitored at 206 nm.

carried out on HPLC by two approaches using biospecific principles. The first one was the cleavage by specific hyaluronidase from bovine testes and analysis of the degradation products by HPLC. The second one was the specific binding of ^{125}I-HABP to the sample and further determination of the changes in the elution radioactive profiles before and after the binding.

The chromatographic profile of the hyaluronidase-digested sample is shown in Fig. 1(b). Comparing this profile with that of the nondigested sample it is clear that the macromolecular peak was completely diminished and transformed to the peaks of low molecular weight products with the retention time about 14 min. This retention time corresponds to the average molecular weight of approximately 9000 Da obtained by Turner *et al.* (1988) who described the same end products of HA after bovine testicular hyaluronidase splitting.

Further high sensitive and specific confirmation of HA identity in the sample was performed by use of ^{125}I-HABP. This marker is widely used in clinical tests for determination of HA in biological fluids such as serum (Tengblad, 1980; Laurent & Tengblad, 1980; Brandt *et al.*, 1987) and synovial fluid (Saari *et al.*, 1990). Our recent results show that the HABP prepared binds to HA with high specificity and no significant binding was observed with other similar polysaccharides such as dextran, polygalacturonic acid and chondroitin sulphate (Orvisky *et al.*, 1990). Our results are in agreement with those of Hardingham & Muir (1972) and Hascall & Heinegard (1974) who have found that HABP does not interact with other HA like polyanions as dextransulphate, DNA and sodium alginate. HPLC elution profiles of ^{125}I-HABP measured before and after incubation with HA (Figs 2(a) and 2(b)) indicate that radioactivity of the iodinated binding protein after the incubation (Fig. 2(b)) was divided into two peaks. The first one represents the ^{125}I-HABP protein bound to the HA sample and the second peak is determined as original unbound ^{125}I-HABP.

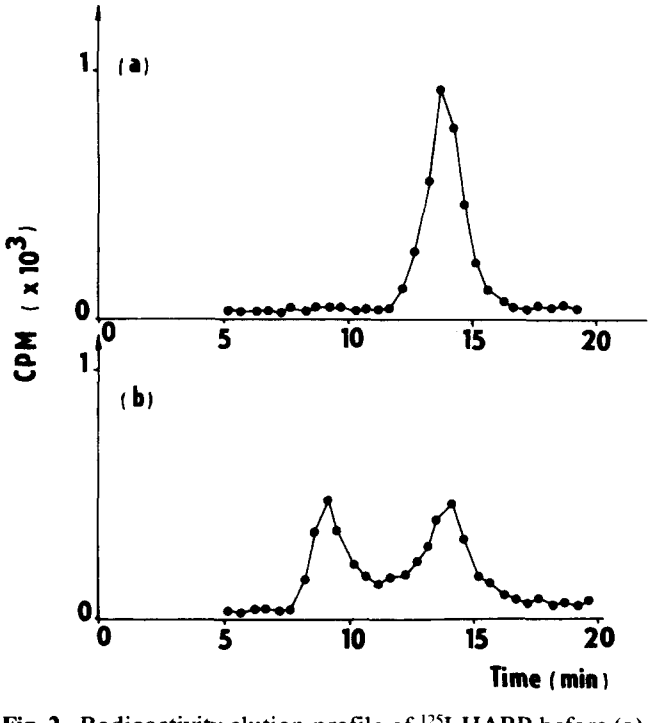

Fig. 2. Radioactivity elution profile of ^{125}I-HABP before (a) and after (b) incubation with the sample. ^{125}I-HABP (100 µl, 82 cpm/µl) and the sample (10 µl, 1 mg/ml) were preincubated for 1 h at 8°C. The samples were chromatographed as previously.

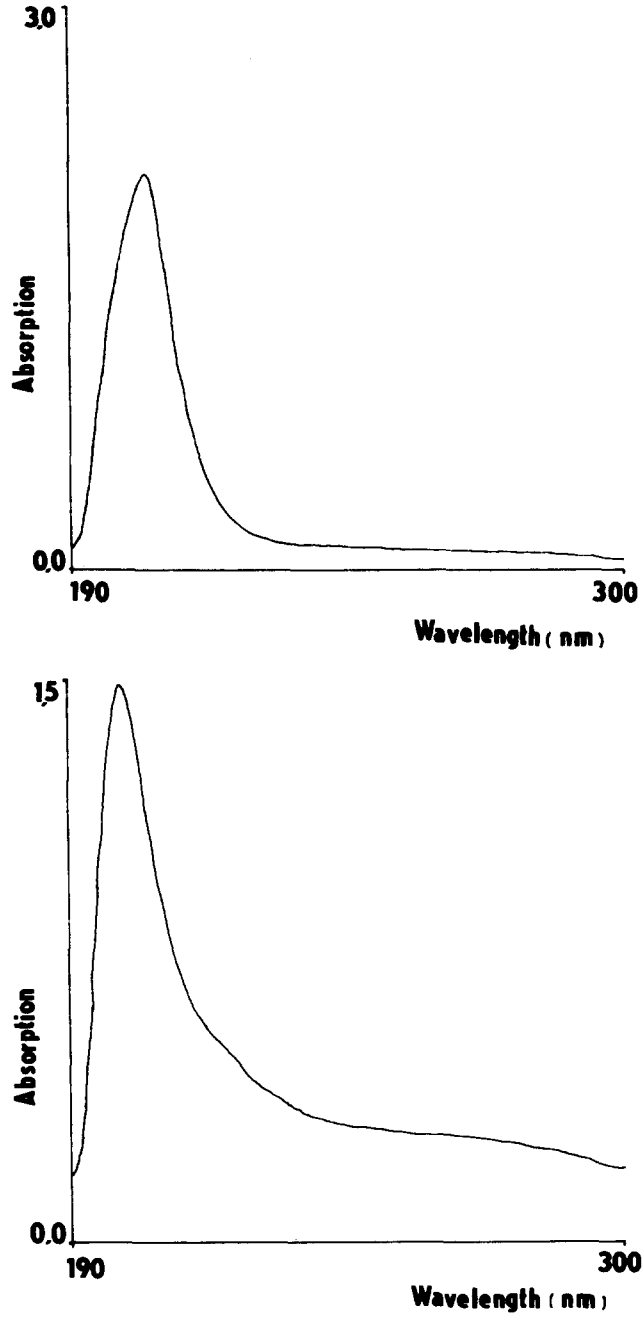

Fig. 3. Ultraviolet spectra of HEALON (a) and the sample of HA from *Streptococcus equi* (b) measured in distilled water at concentrations of 0·5 mg/ml.

Table 1. Protein determination with Coomassie brilliant blue G-2-0 in the HA samples in comparison with the absorption of dextran at the same concentrations. To 1 ml of the sample 1 ml of the protein reagent was added and absorbance at 595 nm was measured after 2 min

	Sample								
	Dextran T-40 (mg)			HA CONTIPRO (mg)			HA HEALON (mg)		
	0·5	1·0	5·0	0·5	1·0	5·0	0·5	1·0	5·0
A_{595}	0·008	0·014	0·017	0·054	0·110	0·389	0·012	0·015	0·026

These measurements supported almost unambiguously the suggestion that the macromolecular component of the sample is HA. Further evidence follows from the comparison of ultraviolet spectra of the sample with known HA standards (HEALON, Pharmacia) — Fig. 3. The HEALON (Fig. 3(a)) and the sample (Fig. 3(b)) spectra in the ultraviolet region are almost identical, this confirms the identification of the sample as HA.

Ultraviolet spectroscopy is also used to estimate the presence of impurities in the HA sample, such as nucleic acids (λ_{max} = 257 nm) and proteins (λ_{max} = 280 nm) (Balazs, 1979). A higher background of the sample spectrum at 280 nm suggested that the sample hyaluronic acid probably contains protein impurities. Their determination was performed by use of the Coomassie brilliant blue absorption method (Sedmak & Grossberg, 1977). The results are shown in Table 1. Carbohydrates, including HA, had almost no influence on the method used since only a little increase in the absorbancy at 595 nm was observed if DEXTRAN-T-40 or HEALON were applied at the sample concentrations. The average concentration of the protein impurities was determined from a calibration curve using bovine serum albumin as standard. The protein content is about 50·5 μg/mg (5·1%) of the sample.

The procedure presented here seems to be a simple algorithm for identification and partial characterization of HA in complex mixtures of industrially-produced HA. The method does not require the isolation and purification of the HA from the mixture and therefore, it is quite suitable for evaluation of the whole purification procedure. We have demonstrated the use of the method on an identification and characterization of HA isolated from cultivation medium of *Streptococcus equi*. We have determined easily the main parameters of technological interest — purity and molecular weight of HA in the sample. A high sensitive determination of HA concentration in complex mixtures is also possible by similar methods, as we have shown elsewhere (Orviský *et al.*, 1990).

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