A constitutive heparinase in a *Flavobacterium* sp.[1]

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Summary. The isolation of a small gram-negative rod, lacking flagella or pili, and tentatively identified as a *Flavobacterium* sp., giving exceptionally high yields of heparinase, is reported. The enzyme was partially purified.
Key words. Heparinase; constitutive heparinase; *Flavobacterium*.

Heparinase (Heparin lyase, E. C. 4.2.2.7.) is an eliminase which cleaves certain α-glycosidic linkages in heparin, an αβ-linked sulphated polisaccharide[4]. Interest has recently been expressed in this enzyme for use in an immobilized enzyme filter for extracorporeal deheparinization of blood[5]. *Flavobacterium heparinum* (ATCC 13125) produces heparinase only when heparin is added to the growth medium as an inducer[6,7]. However, it has been reported that yields of 30–50% of the inducible level can be produced when the organism is grown in a medium deficient in sulphate[7]. Heparinase is also produced by certain anaerobic bacteria such as *Bacteroides* spp.[8] and *Eubacterium* spp.[9] and probably by certain fungi, notably *Aspergillus* spp.[10]. The limitations of present procedure to produce heparinase are the relatively low yields obtained and the expense involved.

In view of these difficulties and the current interest in heparinase for extracorporeal deheparinization of blood, we screened a series of organisms isolated from soil for heparinase activity. We report here the isolation of an organism giving exceptionally high yields of heparinase (a 10-fold higher production as compared to *F. heparinum* ATCC 13125 under the same conditions) and in which the enzyme appears to occur in a constitutive form. The bacterium was isolated from soil by using conventional enrichment methods. Subsequent purification was carried out on a medium consisting of tryptose broth (Difco), solidified with 1.5% agar (Difco). The ability of the organism to degrade heparin was demonstrated by the method of Joubert, Van Rensburg and Pitout[11]. In addition to heparin, the organism also degrades chondroitin sulphate.

The bacterium is a small gram-negative rod, lacking flagella or pili, and the colonies on agar exhibit a faint yellow color. The organism grows optimally at 28–30 °C, but growth and heparinase production still occur at 37 °C. At 42 °C there is no growth. Very poor growth of the organism takes place in the absence of both glucose and yeast extract, although heparinase was still produced. The organism has been tentatively identified as a *Flavobacterium* sp.

Heparinase activity was determined by measuring the disappearance of heparin as judged by metachromasia of Azure A dye[6]. One unit of activity (U) is defined as the amount of enzyme that can degrade 1 mg of heparin completely/h at 37 °C and pH 7.0. Specific activity (SA) is given as units/mg protein. Protein was determined by the method of Lowry et al.[12].

Mass cultures for enzyme extraction were grown on the surface of Roux flasks containing 150 ml of solid medium consisting of trypticase soy broth plus dextrose (BBL) to which had been added 1 g yeast extract (Difco)/l. Enzyme production could be doubled by growing the cells in liquid culture in a fermentor in a medium consisting of glucose (Holpro) 0.2%, trypticase soy broth without glucose (BBL) 0.3%, $MgCl_2$ (Merck) 0.01% and K_2HPO_4 (Merck) 0.25%. The pH after sterilization was 7.6. The addition of heparin was found to be unnecessary for heparinase production. The Roux flasks were incubated at 28 °C. After 48 h the cells were harvested, washed twice in 0.01 M phosphate buffer (pH 7.0) and resuspended in the same buffer in a ratio of 1 g of cells (wet wt)/5 ml buffer. The suspension was sonicated at 4 °C in a Soniprep 150 sonicator (6 × 15 s pulses) and centrifuged at 48,400 × g for 20 min in a Beckman model J 2-21 centrifuge at 4 °C. Heparinase was partially purified by means of hydroxylapatite chromatography followed by chromatofocusing. All steps were carried out in a cold room at approximately 5 °C. The crude extract was adsorbed on a hydroxylapatite column (10 × 2.5 cm, Bio-Gel HTP), prepared and equilibrated in a 0.05 M sodium phosphate buffer (pH 7.0) according to the method in Bio-Rad Labs., California, USA. After washing the column with the same buffer to remove impurities, the enzyme was eluted in a single peak with 0.22 M NaCl-0.05 M phosphate buffer (pH 7.0). The eluate was dialyzed overnight against 0.025 M ethanolamine-acetic acid buffer, pH 7.4, followed by chromatofocusing on a Polybuffer exchanger (PBE 94 column, 0.9 × 15 cm; Pharmacia Fine Chemicals, Uppsala, Sweden), equilibrated with the ethanolamine-acetic acid buffer and eluted with ampholyte Polybuffer 96-acetic acid solution adjusted to pH 6.0. The fraction with enzyme activity was located at a pH of 8.4–8.8.

The enzyme preparation thus obtained was then subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis[13]. At least 4 bands could be seen indicating that partial purification had been obtained. The results of the purification procedure of heparinase from cells grown on solid medium are summarized in the table. Details about the organism will be published elsewhere.

Partial purification of a constitutive heparinase from a *Flavobacterium* sp.

Purification step	Total protein (mg)	Total units (U)	Specific activity	Activity (%)
Cell homogenate	563	4534	8	100
Hydroxyl-apatite	81	4160	514	92
Chromato-focusing	4.9	2018	412	42

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