Regulation of Heparinase Synthesis in *Flavobacterium heparinum*

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Abstract. The effect of various carbon, nitrogen and sulfur sources on the production of heparinase by *Flavobacterium heparinum* in defined medium in the presence and absence of heparin as the inducer has been studied. Carbon catabolite repression has been observed in defined medium containing one of several carbon sources including simple sugars, alcohols and organic acids. Fed batch fermentations result in 10 g/l of cells and heparinase titers as high as 100,000 U/l by avoiding carbon catabolite repression. Growth on heparin as a sole carbon source resulted in both a high growth rate of 0.12 h⁻¹ and a high specific activity of 18 U/mg. Specific heparinase activity was markedly reduced when the end products of heparin catabolism were used as carbon, nitrogen or sulfur sources in defined medium. In defined medium with a low sulfate concentration, of less than 10⁻³ M, specific activities as high as 8 U/mg have been observed even in the absence of the normally required inducer, heparin.

Introduction

Heparinase (E.C. 4.2.2.7) is an inducible cell associated enzyme produced by *Flavobacterium heparinum*. Heparinase acts sequentially, along with other flavobacterial enzymes, to catabolize heparin (Dietrich et al. 1973) as shown in Fig. 1. Recent results from this laboratory (Galliher et al. 1981) described a defined growth medium which enhanced the productivity of heparinase 640-fold over that achieved by previous workers (Linker and Hovingh 1972). Interest in the production of heparinase relates to its use for studies involving the elucidation of heparin structure (Linhardt et al. 1982a and Lindahl et al. 1979), blood deheparinization (Langer et al. 1982a), enzymatic assays for heparin (Kanwar and Farquhar 1979) and the preparation of new, heparin derived anticoagulants (Linhardt et al. 1982a).

The availability of a defined medium for *F. heparinum* facilitates the examination of factors affecting heparinase production. In this paper, results of studies on the effect of carbon, sulfur and nitrogen sources on heparinase production and regulation by the end products of heparin...
catabolism are reported. Most interesting is the observation that in a low sulfate, defined medium, heparinase is produced at a high level in the absence of the inducer heparin. These results provide a new example of an unusually strong sulfate regulation associated with the production of an enzyme. The low sulfate medium obviates the need for added inducer, thus substantially reducing the fermentation costs of producing heparinase.

Materials and Methods

Chemicals. Heparin, as the sodium salt, from porcine intestinal mucosa (Grade II, 153 USP K units/mg), amino acids and 4-nitrocatechol sulfate were from Sigma Chemical Co. Azure A dye was from Fisher Scientific Co. (A-70, certified biological stain, total dye content 70%). All other organic and inorganic chemicals were reagent grade. Media components were obtained from Baltimore Biological Laboratories and Difco Co.

Analytical Determinations. Protein was measured by means of the Biuret reaction (Gornall et al. 1949). The protein concentration of the cells was measured by Biuret assay after sonic disruption (Galliher et al. 1981). Reducing sugars were measured with dinitrosalicylic acid (Miller 1959). Assays were performed using a Gilford model 3723 spectrophotometer (Oberlin, Ohio). Heparinase activity and heparin were measured using an azure A dye assay (Galliher et al. 1981). Because of the polydisperse nature of both the substrate and the product it is not possible to relate the enzymatic activity of the cells to production of an enzyme. The low sulfate medium obviates the need for added inducer, thus substantially reducing the fermentation costs of producing heparinase.

Microorganism. Flavobacterium heparinum, a soil isolate (Payza and Korn 1956) is a gram-negative, non-motile and non-sporing rod. Payza and Korn’s report of motility is noted in Bergey’s Manual (Bergey’s Manual, Eighth Edition, 1974), however, no other reports of motility appear in the literature. We have observed no motility with the ATCC 13125 culture, Linker’s culture (Linker and Hovingh 1972) and our culture, (Galliher et al. 1981). The microorganism was grown at 23 °C in all experiments. The culture was stored on agar slants as previously described (Galliher et al. 1981)

Shake Flask Cultivation. Experiments were conducted in 0.5-l sidearm shake flasks. The defined medium contained (g/l) glucose (stirred separately), 8.0; (NH4)2SO4, 2.0; K2HPO4, 2.5; Na2HPO4, 2.5; MgSO4 • 7H2O (stirred separately), 0.5; L-histidine, 0.2 and L-methionine, 0.2 (stirred separately); heparin, 1.0; trace salts (Na2MoO4 • 2H2O, CoCl2 • 6H2O, MnSO4 • H2O, CuSO4 • 5H2O, FeSO4 • 7H2O, CaCl2 • 2H2O) 1 x 10^-4M and P-2000 antifoam (0.2 ml/l). The shake flasks contained 50 ml of the above medium. L-histidine and L-methionine were added after sterilization with a 0.22 μm Millipore syringe filter. After sterilization the pH of the flasks was 7.8. Shake flasks were inoculated from slants or from a second shake flask with the culture in exponential growth. A 4 to 6 vol.% inoculum was used and the culture was incubated on a 2.5-cm stroke shaker at 200 rpm. Growth was measured by turbidity in a Klett-Summers colorimeter (red filter # 66). A Klett unit to dry cell weight (DCW) conversion factor of 260 was obtained. Growth continued until 400–500 Klett units were achieved during which time the pH dropped to 6.0. The cells were harvested or used to inoculate a fermentor with the above medium with a 4 to 6 vol.% inoculum.

Fermentor Cultivation. The 2-l fermentor (with a 1.5-l working volume) was equipped with controlled agitation, aeration, pH, temperature, and dissolved oxygen. The fermentor medium was the same as above except for KH2PO4 (1.0 g/l) and NaH2PO4 (1.0 g/l). The broth pH was adjusted to 5.0 before steam sterilization for 20 to 25 min at 121 °C. After cooling and inoculation, growth, substrate concentration, heparin concentration and heparinase activity were measured. The aeration rate was approximately 0.5 VVM. The pH was controlled at 7.0 ± 0.2 with 1.0 N NH4OH or 1.0 N H2SO4 addition. Culture harvest was carried out by centrifugation at 4 °C for 10 min at 12,800 g Cell pellets were not washed but were either frozen at −40 °C or sonicated within 30 min of centrifugation. Whole cells or crude cell extract from sonication were always kept at 4 °C during handling. All stored cells or extract were kept at −40 °C in polyethylene bottles.

Intermittent fed batch fermentations were carried out as stated above except that additional glucose was added from a presterilized stock solution. Each addition raised the glucose concentration by 10 g/l. This was done twice during the fermentation to obtain approximately 10 g/l dry cell weight.

Continuous culture was performed using the same equipment with the addition of two pumps; the dilution rate was maintained constant by controlling the inlet flow of medium and maintaining constant volume.

Results and Discussion

Carbon Regulation of Heparinase Production

A defined medium containing heparin (1.0 g/l) and initial glucose concentrations ranging from 3.4 to 68.5 g/l was used to investigate the effect of glucose on growth. Cultures were grown in shake flasks to between 140 and 200 Klett units, harvested and assayed for heparinase activity. A nearly linear decrease in initial growth rate from a maximum of 0.26 h^-1 with 3.4 g/l of glucose to 0.0 h^-1 with 68.5 g/l glucose was observed. The growth lag period also increased with increasing glucose concentration. The specific activity of the heparinase obtained was independent of the initial glucose concentration and averaged 13 ± 3.3 U/mg of cell protein (1 U = 1 mg of heparin degraded per hour).

To overcome the adverse effect of high glucose concentration on the growth rate of F. heparinum a fed-batch fermentation with defined medium was conducted to maintain glucose concentrations below 15 g/l. The