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The Release of Heparinase from the Periplasmic Space of *Flavobacterium heparinum* by Three-Step Osmotic Shock

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ABSTRACT

Heparinase was released from the periplasmic space of *Flavobacterium heparinum* by three-step osmotic shock procedure. The procedure involves resuspending exponentially growing cells consecutively into (1) 40% sucrose, (2) 10 m*M* sodium phosphate, 2 m*M* magnesium chloride, pH 7, and (3) 10 m*M* sodium phosphate, 300 m*M* sodium chloride, 2 m*M* magnesium chloride, pH 7. Typically, 50–75% of the total heparinase activity is recovered by this procedure with an observed 7–15-fold increase in purity. The majority of heparinase activity is released in the final step of the procedure allowing for resolution from cytoplasmic and nonspecific periplasmic material. *F. heparinum* cells can be stored in 40% sucrose at 4°C for up to one week without significant losses in recovery yields.

Index Entries: Heparinase; periplasmic protein; osmotic shock; purification; *Flavobacterium*.

INTRODUCTION

Heparinase (EC 4.2.2.7), an inducible enzyme produced by *Flavobacterium heparinum*, catalyzes the first step in the heparin catabolic pathway

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of this organism (*1*). Heparinase is an α-eliminase (*2*) that cleaves heparin at α-glycosidic linkages in heparin's major repeating unit → 4)-α-deoxy-2-sulfamino-α-D-glycopyranose 6 sulfate-(1→4)-α-L-idiopyranosyluronic acid 2-sulfate-(1→) (*3*). This enzyme has been used in several applications, including structural determinations of heparin (*4*) and the preparation of low-mol wt heparin anticoagulants (*5*), antitumor agents (*6*), and anti-atherosclerotic therapeutics (*7*).

Recent emphasis has focused on the development of an immobilized enzyme filter for blood deheparinization (*8,9*). The construction and testing of this device has created a demand for greater quantities of heparinase while the potential for eventual clinical implementation places further requirements on desired levels of purity. In attempting to establish *F. heparinum* as a reliable source for this enzyme, nutritional studies were carried out that identified the unique heparin induction/sulfate repression regulation of this enzyme, leading to the development of a fermentation strategy (*10,11*). The enzyme is reported to have a mol mass of 42,000 daltons with an isoelectric point value of 8.5 (*12*). However, the recovery of heparinase from *F. heparinum* has thus far been unsuccessful in providing material of the quanitity and purity desired for a clinical device.

In this work, an attempt was made to develop a recovery process based on the presumed physiological location of heparinase in the periplasmic space of *F. heparinum*. An adaptation of the osmotic shock method reported by Neu and Heppel (*13*) was successful in preferentially releasing heparinase from exponentially growing cells. An examination of various parameters affecting the extent of heparinase released by osmotic shock was also carried out.

MATERIALS AND METHODS

Chemicals

The sodium salt of heparin from porcine intestinal mucosa was obtained from Hepar Industries, Franklin, OH. Azure A dye was from Fisher. Bio-Rad dye reagent and chemicals for SDS-polyacrylamide gel electrophoresis were purchased from Bio-Rad. All other reagents were reagent grade. Water was MilliQ (Millipore) purified.

Strain and Growth Conditions

The heparinase producing strain of *F. heparinum* used in this work was a generous gift of Alfred Linker (Veterans Administration Hospital, Salt Lake City, UT). This bacterium is a rod-shaped Gram negative faculative anaerobe with a characteristic yellow pigmentation.

In these experiments, the organism was grown at 30°C in 2.8-L shake flasks containing 500-mL defined medium. The defined medium consisted of: K_2HPO_4, 3 g/L; $KH_2PO_4 \cdot H_2O$, 1.5 g/L; NaCl, 0.5 g/L; NH_4CL, 1.0 g/L; $MgSO_4 \cdot 7H_2O$, 10 m*M*; L-histidine, 0.2 g/L; L-methionine, 0.2 g/L; glucose, 8.0 g/L; heparin, 1.0 g/L; and 10^{-4} *M* each of $NaMoO_4 \cdot 2H_2O$, $CoCl_2 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$, and $CaCl_2$.

The organism can be stored for up to 2 wk on agar plates (1.5% Difco agar in defined medium containing heparin, 4 g/L as the sole carbon source) or indefinitely at −70°C in defined medium containing 10% DMSO.

Analytical Determinations

Heparinase activity was assayed by observing the metachromatic shift of azure A from blue to red in the presence of heparin, according to the procedure of Galliher et al. (*14*). The change in absorbance was measured at 620 nm in the linear range of the assay and compared with a standard curve of 0–8 mg/mL heparin in assay buffer (0.25*M* NaOAc, 0.0025*M* CaOAc, pH 7). One unit of activity by this assay corresponds to the amount of enzyme that degrades 1 mg of heparin/h. Alternately, heparinase activity was determined by measuring the increase in absorbance at 232 nm caused by the formation of double bonds in the breakdown products as compared to a standard curve of completely degraded heparin. Culture samples measured by this assay were prepared by complete sonication (Branson W-350 Sonifier, 50% pulsed, #6 power level, 45 s on/30-s off cycles for 24-min cell concentration of 5×10^{10} cells/mL), protamine sulfate (2 mg/mL) precipitation of nucleic acids, and centrifugation to remove cell debris.

β-Galactosidase activity was determined by the method of Miller (*15*). Protein content was measured by the Bio-Rad protein assay (*16*). Growth of the organism was monitored by measuring the absorbance of cell suspensions at 600 nm.

Osmotic Shock Procedure

Osmotic shock procedures were carried out by exposing *F. heparinum* cells sequentially to three different solutions. These were (a) an osmotically stabilizing solution, sucrose or glucose, in 10 m*M* sodium phosphate buffer and two osmotically "non-stabilizing" solutions; (b) 10 m*M* sodium phosphate (low-salt buffer); and (c) 10 m*M* sodium phosphate, 150–300 m*M* NaCl (high-salt buffer). The cells were initially harvested and subsequently removed from each solution by centrifugation (7000*g*, 10 min) in a Sorval RC2-B refrigerated centrifuge. Unless otherwise stated, all procedures were carried out at pH 7 and 4°C, at cell concentrations of 5×10^{10} cells/mL. An aliquot of each culture was sonicated and used as the 100%

control. Supernatants from the osmotic shock solutions as well as sonicates were dialyzed in 10 m*M* sodium phosphate, 150 m*M* NaCl prior to evaluation for enzymatic activity and protein content.

RESULTS

Release of Heparinase from *F. heparinum*

An initial attempt to recover heparinase from *F. heparinum* employed a variation of the osmotic shock procedure of Neu and Heppel (*13*). *F. heparinum* were grown to midexponential phase in defined medium supplemented with 0.2 g/L lactose, to induce β-galactosidase production. The cells were harvested by centrifugation and resuspended in 20% sucrose. EDTA, 20 mM, was added as a disruptive agent. This treatment was followed by resuspension of the "disrupted" cells into an osmotically nonstabilizing buffer; 10 m*M* phosphate, pH 7 (low salt). Less than 5% of the total herparinase activity was detected in the supernatant of this low-salt wash. It has been reported that a second wash with an osmotically nonstabilizing solution enhances product recovery (*17*). In the preliminary experiment, a second wash with a phosphate buffered solution containing 150 m*M* sodium chloride was carried out. Heparinase is most stable in the presence of this level of sodium chloride, which was included to prevent potential denaturation (*12*).

Heparinase appears to be released preferentially into the high-salt solution as evidenced by an increase in specific activity over that of the whole cells (sonicate control). The small quantity of β-galactosidase activity detected in the recovery solutions indicates that little cytoplasmic material is released by this procedure (Table 1).

Alternate methods of cell disruption, including treatment with toluene, chloroform, and lysozyme, and light sonication were examined. Both toulene and chloroform were unable to liberate heparinase from *F. heparinum* cells, whereas the addition of lysozyme did not increase the extent of release over the sucrose/EDTA treatment. Light sonication was capable of preferentially releasing heparinase, but is difficult to control.

An experiment was carried out in which osmotically stabilized cells were divided into two equal batches and resuspended separately into low- and high-salt solutions. After this initial treatment, the cells were divided again into two batches and resuspended separately in the two nonstabilizing solutions. All supernatants were collected and assayed for heparinase activity and protein content. Data from this experiment (Table 2) demonstrates that the order of solutions used in the three-step treatment is not only advantageous but necessary for enzyme recovery. Exposing osmotically stabilized cells immediately to a high salt solution results in a poor release of protein, potentially owing to the osmotic protection offered by 150 m*M* NaCl. Additionally, replacing the third step of

Table 1
Specific Release of Heparinase from *F. heparinum* by Three Step Osmotic Shock Treatment

sample	heparinase recovery (%)	heparinase sp. act. (U/mg)	β-galactosidase recovery (%)
sonicate	100.0	29.2	100.0
20% sucrose	0.0	0.0	2.0
10 mM sodium phosphate (low salt)	4.5	26.2	0.7
10 mM sodium phosphate 150 mM NaCl (high salt)	39.8	83.2	0.7

Table 2
Dependence of Heparinase Release on the Order of Washing *F. heparinum* Cells with Buffers of Different Ionic Strengths

sample	heparinase recovery (%)	sp. act. (U/mg)	protein recovery (%)
sonicate	100.0	26.0	100.0
I:low salt	12.8	38.1	8.7
II:low salt	9.0	65.4	3.6
high salt (150 mM Na^+)	42.7	216.6	5.1
I:high salt (150 mM Na^+)	1.4	24.0	1.5
II: low salt	3.0	76.0	1.0
high salt (150 mM Na^+)	4.6	233.0	0.5

the procedure with a low-salt solution wash fails to release heparinase comparable to that released into a high-salt solution. The order of solutions did not have a significant impact on the specific activity of released heparinase, only the yield.

This three-step osmotic shock treatment has been carried out several times, leading to some general observations. The amount of protein released into each of the recovery solutions is approximately equal, 4–9% of the total cell protein. At pH 7, heparinase is preferentially released into the high-salt recovery solution containing the majority of the total activity released, with a typical 7–15-fold increase in specific activity. The effects of operational parameters on heparinase release were systematically investigated.

Effect of EDTA

The quantity of EDTA, added as the disruptive agent in the initial step of the osmotic shock treatment, was varied from 0 to 20 m*M*. Data from this experiment (Table 3) indicate that the amount of heparinase released into the high-salt recovery solution does not depend on the

Table 3
Effects of Operational Parameters on the Recovery of Heparinase from *Flavobacterium heparinum* by the Three Step Osmotic Shock Procedure

parameter varied	parameter value	sample	recovery (%)	sp. act. (U/mg)
EDTA	0 mM	low salt	17.8	N.D.
		high salt (150 mM Na^+)	45.2	
	1mM	low salt	15.8	
		high salt (150 mM Na^+)	48.0	
	2 mM	low salt	10.0	
		high salt (150 mM Na^+)	46.3	
	5 mM	low salt	11.3	
		high salt (150 mM Na^+)	52.1	
	10 mM	low salt	15.5	
		high salt (150 mM Na^+)	47.7	
	20 mM	low salt	24.8	
		high salt (150 mM Na^+)	48.9	
sucrose	10 %	low salt	3.3	15.3
		high salt (300 mM Na^+)	46.6	158.6
	20 %	low salt	5.6	14.6
		high salt (300 mM Na^+)	58.2	104.8
	40 %	low salt	11.3	22.7
		high salt (300 mM Na^+)	79.7	181.7
ionic strenght in high salt buffer	100 mM	high salt	37.9	204.9
	200 mM	high salt	63.7	343.7
	300 mM	high salt	69.5	372.4
	400 mM	high salt	67.0	358.3
	500 mM	high salt	66.8	360.7
Mg^{++}in recovery buffers	0 mM	low salt	3.1	16.8
		high salt (300 mM Na^+)	63.8	341.5
	2 mM	low salt	3.0	15.9
		high salt (300 mM Na^+)	69.5	372.4
pH of recovery buffers	6.0	low salt	5.3	N.D.
		high salt (150 mM Na^+)	20.7	
	6.7	low salt	13.7	
		high salt (150 mM Na^+)	44.8	
	7.5	low salt	17.6	
		high salt (150 mM Na^+)	80.8	
	8.6	low salt	25.3	
		high salt (150 mM Na^+)	68.6	

quantity of EDTA used to disrupt the cells. In fact, it appears that EDTA is not required for the osmotic release of heparinase from *F. heparinum*. Subsequent experiments were carried out without the addition of EDTA.

Effect of Sucrose Concentration

The quantity of sucrose added to osmotically stabilize the cells was varied from 10 to 40%. Typical results for this experiment are shown in Table 3. In repeating this experiment several times (data not shown), it

was observed that the recovery of heparinase from cells exposed to 40% sucrose was consistently greater than 50%, usually within the 75–90% range, whereas recoveries from cells exposed to lesser sucrose concentrations were more variable. Therefore, subsequent experiments were carried out using 40% sucrose in the first step of the procedure.

Effect of Ionic Strength

An experiment was carried out in which the ionic strength of the high-salt recovery buffer was varied from 100 to 500 m*M*. Data from this experiment (Table 3) displays an increase in recovery yield as a function of ionic strength, leveling off at 300 m*M*.

Effect of Magnesium in Recovery Buffers

The inclusion of divalent cations in recovery buffers in certain cases has led to a more selective release of periplasmic material (*17*). An experiment was carried out in which *F. heparinum* cells, osmotically stabilized in 20% sucrose, were divided into two aliquots and separately washed with recovery buffers containing either 0 or 2 m*M* $MgSO_4$ (Table 3). There was no observed increase in specific activity or yield as a result of the presence of magnesium in the recovery solutions. However, cells that were treated with magnesium formed tighter cell pellets and were more readily resuspended in subsequent solutions.

Effect of pH

F. heparinum cells, osmotically stabilized in 20% sucrose, were divided into four equal aliquots and osmotically shocked separately at various pH levels. The results (Table 3) show that the amount of heparinase released into low-salt solutions increases with increasing pH throughout the range tested (6.0–8.6), and that the greatest overall recovery, low- and high-salt fractions, is observed at pH 7.5.

Effect of Growth Rate

The effect of growth rate on the extent of enzyme release was examined. An experiment was carried out in which samples of *F. heparinum* taken during various stages of a culture (Fig. 1) were subjected to the three-step osmotic shock procedure. The results (Table 4) show that the maximal recovery occurs from samples taken during mid to late exponential phase. The specific activity of heparinase released increases throughout exponential growth while the total amount of protein released remains relatively constant. This trend was observed in three independent experiments. The decrease in recovery of heparinase activity during stationary growth phase appears related to a decrease in the amount of protein released rather than a decline in specific activity.

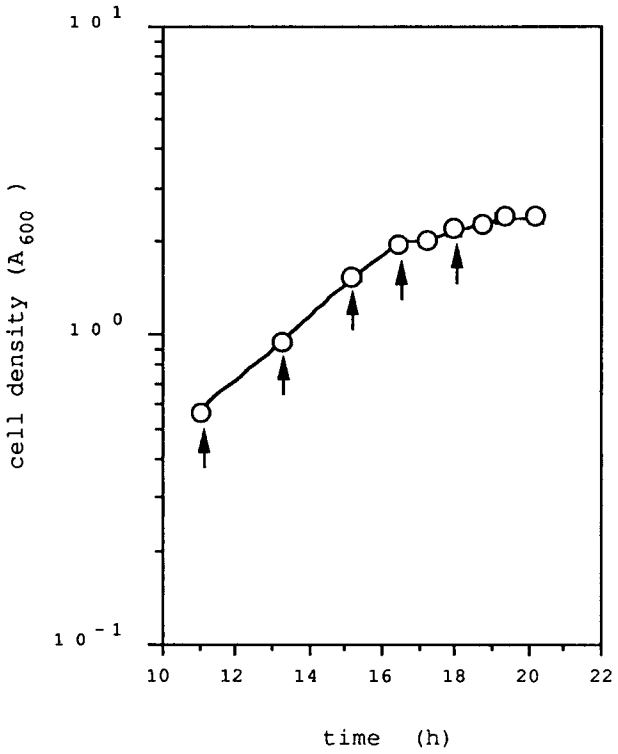

Fig. 1. Profile of *F. heparinum* cell density during a 500-mL shake flask fermentation. Cell density was determined by measuring the absorbance at 600 nm at various times during a *F. heparinum* fermentation (open circles). Arrows indicate times at which an aliquot of the culture was harvested and subjected to the three-step osmotic shock procedure. The first three samples were of exponentially growing cells, where the growth rate was at its maximum of 0.21/h. The last two samples were taken during the onset of stationary growth. Results of the osmotic shock procedure are listed in Table 4.

Recovery from Cells Stored in Sucrose

Two experiments were carried out in which *F. heparinum* cells were stored in sucrose as described in Table 5 for varying lengths of time before being washed with the recovery buffers. The results of these experiments (Table 5) demonstrate that *F. heparinum* cells can be kept in sucrose for at least one week without appreciable loss of heparinase recovery by osmotic shock treatment.

DISCUSSION

This work demonstrates a novel three-step osmotic shock procedure for selectively releasing heparinase from the periplasm of *F. heparinum*. The method differs from classical two-step procedures (*17,18*) in that

Table 4
Release of Heparinase from *F. heparinum*
by Three Step Osmotic Shock During Various Phases of Cell Growth

sample	A_{600}	μ	heparinase recovery (%)	sp. act. (U/mg)	protein recovery (%)
sonicate	0.58	0.21	100.0	16.0	100.0
low salt			1.7	4.3	6.3
high salt(150mM)			11.2	25.0	7.2
sonicate	0.96	0.21	100.0	21.3	100.0
low salt			21.4	31.7	9.5
high salt(150mM)			53.9	96.0	7.9
sonicate	1.55	0.21	100.0	25.0	100.0
low salt			6.3	36.0	4.4
high salt(150mM)			51.0	210.0	6.1
sonicate	1.98	0.13	100.0	28.7	100.0
low salt			7.9	62.5	3.6
high salt(150mM)			21.4	170.0	3.6
sonicate	2.36	0.06	100.0	32.7	100.0
low salt			3.3	40.0	2.7
high salt(150mM)			13.5	163.3	2.7

Table 5
Storage of *Flavobacterium heparinum* Cells in 20% Sucrose (experiment 1) and Stored Frozen at –20°C (experiment 2) in 40% Sucrose Prior to Recovery of Heparinase by Osmotic Shock

	storage time	sample	recovery (%)
experiment 1	0 h	low salt	11.4
		high salt(300 mM Na^+)	39.0
	1 h	low salt	1.4
		high salt(300 mM Na^+)	33.0
	2 h	low salt	9.0
		high salt(300 mM Na^+)	43.0
	4 h	low salt	6.8
		high salt(300 mM Na^+)	38.0
	8 h	low salt	8.3
		high salt(300 mM Na^+)	49.8
experiment 2	0 d	low salt	8.6
		high salt(300 mM Na^+)	34.0
	1 d	low salt	11.0
		high salt(300 mM Na^+)	50.6
	2 d	low salt	9.3
		high salt(300 mM Na^+)	43.2
	4 d	low salt	9.7
		high salt(300 mM Na^+)	55.6
	7 d	low salt	5.6
		high salt(300 mM Na^+)	47.8

osmotically stabilized cells are sequentially washed with two recovery buffers. These are; a low ionic strength buffered solution, 10 m*M* sodium phosphate, followed by a buffered solution containing sodium chloride. Although each of the two recovery solutions contain approximately equal amounts of protein, 4–9% of the total cellular protein, the heparinase activity is found mainly in the high-salt wash. The order of the three steps, sucrose, low salt, and high salt, appears necessary for effective release, and confers the advantage of resolving heparinase from both cytoplasmic and nonspecific periplasmic proteins.

The selective release of heparinase into solutions containing sodium ions may be explained in part by the natural cation exchange capacity of the bacterial cell surface. Heparinase, which has a pI of 8.5 (*12*), may remain associated with osmotically disrupted cells by electrostatic interaction with the negatively charged lipid bilayers. In this case, the increasing concentration of sodium ions may serve to displace heparinase from cell surfaces. It was observed that the amount of heparinase released into "low salt" solutions increases with increasing pH in the range of 6.0–8.5. As the pH is increased in this range, the heparinase molecule becomes less positively charged, and therefore, is less likely to remain electrostatically associated with cell surfaces. The cell membranes should be negatively charged in this range as the pK' of the phospholipid component phosphate groups is in the range of 1–2 (*19*). The observation of this effect may provide a basis for more efficient recoveries of basic periplasmic proteins.

Maximum recovery from cells treated during late exponential phase has been reported for several periplasmic enzymes recovered by osmotic shock treatment (*17*), and therefore, our results were not surprising. However, the observation of increasing specific heparinase activity of material released throughout the exponential growth phase while the total amount of protein released remains relatively constant, was unexpected.

Several operational parameters were investigated to optimize the three-step procedure. The most consistent results were obtained by exposing exponentially growing *F. heparinum* cells consecutively to

1. Sucrose (40%), 10 m*M* sodium phosphate, pH 7;
2. Sodium phosphate (10 m*M*), 2 m*M* $MgSO_4$, pH 7; and
3. Sodium phosphate (10 m*M*), 300 m*M* sodium chloride, 2 m*M* $MgSO_4$, pH 7.2–7.5.

Typically, 50–75% of the total heparinase activity is recovered in the final wash with a 7–15-fold increase in purity.

The storage of *F. heparinum* cells in sucrose also was examined. Recovery yields were not significantly diminished when the cells were kept in sucrose for up to one week. This result contradicts the general mechanistic hypothesis for osmotic shock that plasmolysis is required for effective release of periplasmic material. Plasmolyisis refers to the phenomenon of protoplast

shrinkage and retraction of the cytoplasmic membrane from the rigid cell wall on exposure to an osmotic stabilizing agent (*20*). After a 10–15 min exposure to sucrose, bacterial cells begin to swell and attain their original protoplasmic volume, which is limited by the cell wall itself (*21*). A prolonged exposure to sucrose should therefore limit the degree of volumetric expansion when the cells are transferred to a low osmotic pressure environment and a rapid inflow of water occurs, thereby diminishing the expulsion of periplasmic material. Perhaps the cell wall of *F. heparinum* is less rigid, allowing for the leakage of periplasmic material with less abrupt environmental changes, or that another mechanism accounts for the observed release of periplasmic material from these cells. In any case, the ability to temporarily store *F. heparinum* cells in sucrose potentially facilitates the processing of larger samples for heparinase recovery by this method.

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