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Improvement of production, assay and purification of streptavidin

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SUMMARY

The production of streptavidin by *Streptomyces avidinii* in several different media was examined at 24, 48 and 72 hours. Flask studies indicated that fermentation media containing either complex or multiple carbon sources resulted in higher yields of streptavidin than media with a single carbon source. Streptavidin could be detected in crude fermentation broths by use of a tritiated biotin binding assay. This assay appears to give useful estimates of streptavidin production. Depending upon the medium employed, streptavidin yields ranged from 0.5 mg/l to 53 mg/l. Production was successfully scaled up to ten liter fermentors. Streptavidin was purified in a one step process from centrifuged, concentrated fermentation broths by binding the protein to an iminobiotin column at pH 11 followed by elution at pH 4.0. Recovery percentages varied depending upon the solubility of the fermentation media ingredients.

INTRODUCTION

Streptavidin, secreted by *Streptomyces avidinii*, is a nonglycosylated neutral protein [1,2], that binds four biotins as tightly as egg white avidin. Biomedical researchers find many uses for biotin-avidin systems [3,4] but encounter nonspecific binding of the positively charged, glycosylated egg white avidin to negatively charged membranes and plastics [2].

Because our diagnostic division required large quantities of streptavidin for developing detection reagents [5] at a time when limited amounts were commercially available, we evaluated methods for producing high concentrations in a variety of fermentation media.

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Although several methods have been published for quantitation of avidins [3], the heterogeneity of media composition and the production of dark pigments by *Streptomyces avidinii* have rendered a spectrophotometric assay difficult without prior purification of the fermented culture media. Since purification may result in variable yields, we adapted a radioactive biotin-binding method [6] to measure streptavidin directly in crude fermentation broths. This method enabled us to screen a variety of fermentation media for optimal streptavidin production. We have also simplified a published method for isolating streptavidin from culture supernatants using an iminobiotin column [7].

MATERIALS AND METHODS

A. Growth and fermentation of Streptomyces avidinii

1. Culture and inoculum preparation: *Streptomyces avidinii* ATCC 27419 was the streptavidin producing strain used in this study [8]. A one cm square agar block of *S. avidinii* spores and mycelia, cut from a 10 day slant culture grown on starch-casein [9] medium at 28°C, served as the inoculum for the germination medium [8]. The germination medium (medium F, Table 1) was dispensed in 50 ml aliquots into 250 ml Erlenmeyer flasks with cotton gauze plug. After 72 h growth at 28°C, five percent (v/v) of the germination growth material was transferred to the fermentation media.

2. Fermentation flask studies: The nine fermentation media tested for optimal production of streptavidin by *S. avidinii* are listed in Table 1. Media varied in carbon and nitrogen sources, and in degree of complexity; i.e. single, multiple or complex carbon sources. The pH values of the media were between 6.4 and 7.0 after autoclaving. Fermentation media were dispensed as 50 ml aliquots per 250-ml Erlenmeyer flasks with cotton gauze plugs. All flasks were incubated at 28°C in a New Brunswick Psychotherm Rotary Shaker set at 200 rpm, with no humidity control. Flasks were harvested at 20, 48 and 72 h. Wet weights of cell mass were determined by weighing the cells after centrifugation at 6000 × *g* for 20 min. Cell weight was not determined for medium B, due to the presence of $CaCO_3$.

B. Fermentor study

A Chemap ten-liter fermentor was prepared containing nine liters of medium C without glucose. Filter-sterilized glucose was added to the sterile medium through a port to a final concentration of 1%. The glucose concentration was maintained at 1% throughout the run. The fermentor was inoculated with 3.9% v/v of *S. avidinii* cells grown in germination medium.

Previous flask studies indicated that optimal production of streptavidin occurred between 28 and 30°C and at pH 6.5, therefore, fermentor conditions were set at 28°C, 410 rpm, pH control at 6.5, and air at one liter/min. Aseptic 20 ml samples were taken daily for biotin binding assays. The fermentor growth material was usually harvested at 72 h, depending on the biotin binding results. Fermentor contents were centrifuged and the supernatant was decanted and filtered through two layers of Whatman #54 filter paper. The light golden brown supernatant, containing the desired protein, was either purified immediately or frozen at −70°C. Two fermentor runs are compared in Fig. 1.

C. Assay of streptavidin with the tritiated biotin binding assay

A sample of culture medium was centrifuged to remove cells and debris. The supernatant could be used directly, or diluted into 0.1% bovine serum albumin in phosphate buffered saline (PBS = 130 mM NaCl, 15 mM KH_2PO_4, 8 mM Na_2HPO_4, 3 mM KCl, pH 7.4). The sample was incubated at room temperature for thirty minutes with an excess of tritiated biotin (New England Nuclear 40 Ci/mmol, labeled in positions 8 and 9). The total volume of the incubation mixture was 200 microliters: 150 microliters of the mixture was PBS. The mixture was fractionated on a G-25 Sephadex column (Pharmacia). One milliliter fractions were collected and 100 microliter samples counted in 10 milliliter PCSII scintillation fluid (Amersham). As shown in Fig. 2, the biotin that was bound to large molecules eluted from the Sephadex column early and the un-

Table 1

Fermentation media

A.	Glucose	10.0	g [8]	B.	Soybean meal (MS stock)	30.0	g
	Yeast extract (Difco)	0.5	g		Distillers soluble [14]	7.5	g
	DL-Asparagine	1.0	g		Cerelose	20.0	g
	K_2HPO_4	0.1	g		$CaCO_3$	10.0	g
	$MgSO_4 \cdot 7H_2O$	0.5	g		NaCl	2.5	g
	$FeSO_4 \cdot 7H_2O$	0.01	g		Di* H_2O	1.0	l
	Di* H_2O	1.0	l		pH	7.0	
	pH	6.8					
C.	Glucose	10.0	g [7]	D.	Glucose	10.0	g
	Tryptone (Difco)	5.0	g		Peptone (Difco)	5.0	g
	Yeast extract (Difco)	3.0	g		Yeast extract (Difco)	5.0	g
	Malt extract (Difco)	3.0	g		Beef extract (Difco)	3.0	g
	Di* H_2O	1.0	l		Soluble starch (Baker)	24.0	g
	pH	6.4			Di* H_2O	1.0	l
					pH	6.4	
E.	Corn steep liquor (Corn Products Corp)	40.0	ml	F.	NZ amine (type A) (Sheffield Products)	10.0	g
	Sucrose	10.0	g		Beef extract (Difco)	3.0	g
	Glucose	10.0	g		Glucose	10.0	g
	$MgSO_4 \cdot 7H_2O$	0.25	g		NaCl	5.0	g
	KH_2PO_4	0.5	g		Di* H_2O	2.0	l
	$NaNO_3$	3.0	g		pH	6.95	
	$ZnSO_4 \cdot 7H_2O$	0.1	g				
	$CaCO_3$	0.25	g	G.	Soybean meal (MS stock)	15.0	g
	Di* H_2O	1.0	l		Dehydrated potatoes (Bonnie Hubbard)	15.0	g
	Adjust pH	7.0			Glucose	10.0	g
H.	Corn steep liquor (Corn Products Corp)	25.0	g		$CoCl_2 \cdot 6H_2O$ (0.05% sol)	10.0	ml
	Soluble starch (Baker)	15.0	g		$NaHCO_3$	10.0	g
	$CuSO_4 \cdot 5H_2O$	0.005	g		**L-20 salts	2.0	ml
	$MnCl_2 \cdot 4H_2O$	0.005	g		Di*H_2O	1.0	l
	$ZnSO_4 \cdot 7H_2O$	0.005	g		pH	7.0	
	$CaCO_3$	4.0	g				
	Di* H_2O	1.0	l	I.	NZ amine (type A) (Sheffield Products)	20.0	g
	pH	6.75			Glucose	10.0	g
					KH_2PO_4	0.6	g
					K_2HPO_4	1.4	g
					$ZnSO_4 \cdot 7H_2O$	0.0025	g
					$CaCl_2 \cdot 2H_2O$	0.0025	g
					$MgSO_4 \cdot 7H_2O$	0.0025	g
					$FeSO_4 \cdot 7H_2O$	0.0025	g
					Di* H_2O	1.0	l
					pH	7.0	

* Distilled H_2O.

** L-20 Salts ingredients are as follows:

85% (w/w) H_3PO_4	33.88 ml	0.1 M $CuSO_4 \cdot 5H_2O$	1.0 ml
96% (w/w) H_2SO_4	16.7 ml	0.1 M $CoCl_2 \cdot 6H_2O$	1.0 ml
KCl	22.4 g	0.1 M $Na_2MoO_4 \cdot 2H_2O$	1.0 ml
$MgCl_2 \cdot 6H_2O$	25.4 g	0.5 M H_3BO_3	1.0 ml
1.0 M $MnSO_4 \cdot H_2O$	1.0 ml	Di* H_2O up to	700.0 ml
0.1 M $ZnSO_4 \cdot 7H_2O$	1.0 ml	$CaCl_2 \cdot 2H_2O$ in 300 ml Di* H_2O	1.47 g

This solution is kept refrigerated and in the dark.

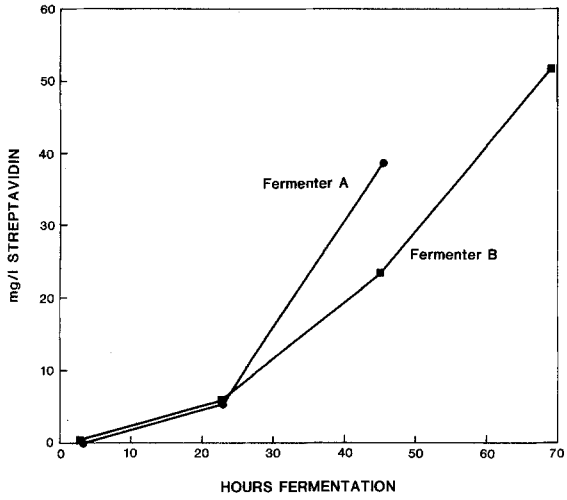

Fig. 1. Production of streptavidin in 10-l fermentors.

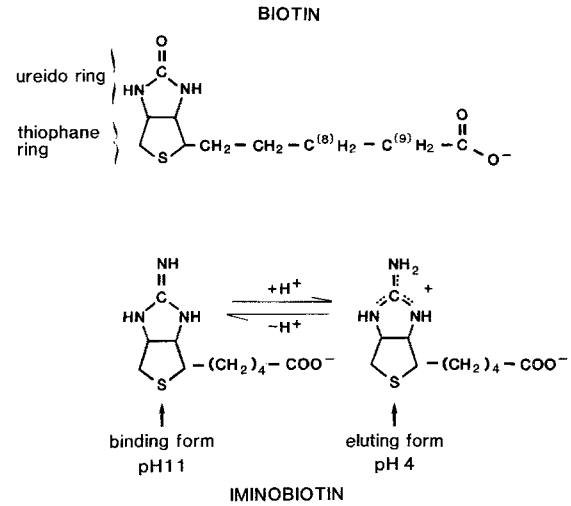

Fig. 3. Structure of biotin and iminobiotin.

bound biotin separated reasonably well from the protein bound peak. From the total radioactivity eluted in the first peak and the specific activity of the tritiated biotin, the amount of streptavidin could be calculated.

D. Protein purification

1. Concentration: The supernatant from the centrifuged culture was concentrated tenfold at 4°C with an Amicon hollow fiber device fitted with a reservoir to facilitate processing. Our best results were obtained with a 10 000 M.W. cutoff filter. The filtrate appeared as a pale yellow fluid and the concentrate was dark brown. The filtrate had no streptavidin detectable by the biotin binding assay, whereas the concentrate retained 90% of the streptavidin assayed in the supernatant.

2. Iminobiotin column: Iminobiotin is a biotin analog (Fig. 3) that binds avidins at pH 11, but not at pH 4 [7]. At high pH, the binding portion of the

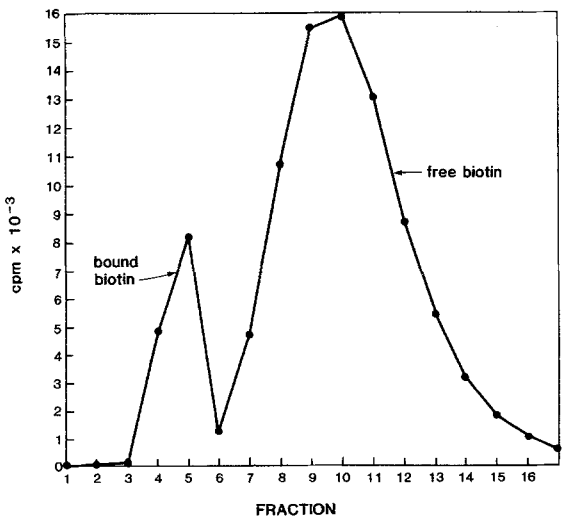

Fig. 2. Assay separation of streptavidin bound ^{3}H-biotin from free biotin on G-25 Sephadex.

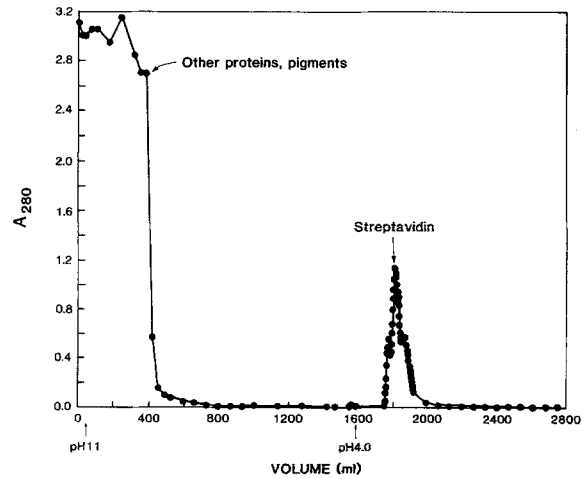

Fig. 4. Affinity purification of streptavidin from concentrated culture supernatant on an iminobiotin column.

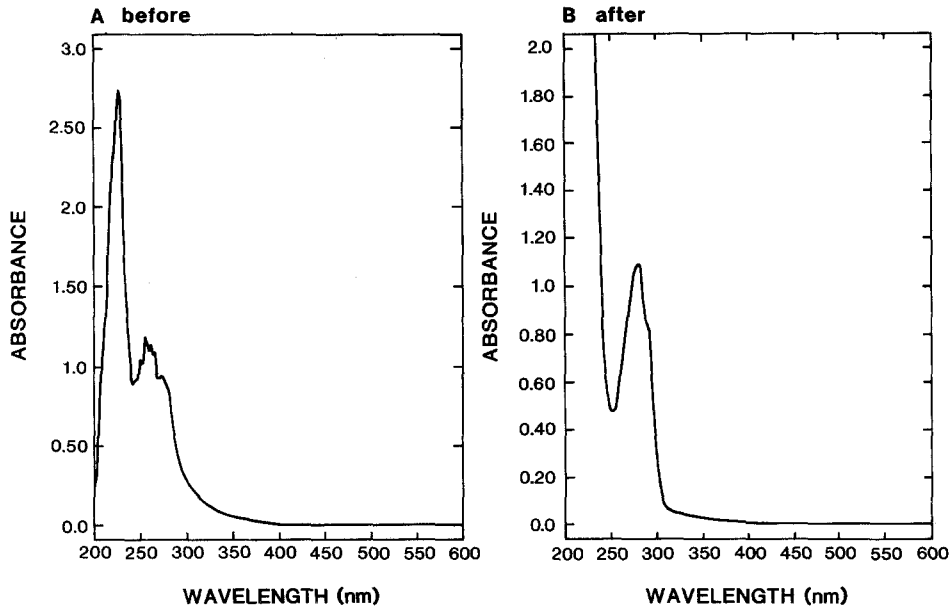

Fig. 5. Spectra of affinity-purified streptavidin before and after acetic acid treatment.

iminobiotin molecule is uncharged, like biotin, but at low pH the imine acquires a positive charge which lowers its affinity to avidin by several orders of magnitude. Pierce (Rockford, IL) provides immobilized iminobiotin in gel form with a reported capacity of one mg avidin/ml resin. A 2.5 cm diameter by 25 cm column was packed with 90 ml of iminobiotin gel and equilibrated with 50 mM ammonium carbonate at pH 11. The culture concentrate was diluted 1:1 with 100 mM ammonium carbonate and the pH was kept at 11. Approximately 20 mg of streptavidin was applied to the column, which was washed with pH 11 buffer until the eluate ceased to absorb at 280 nm. The column buffer was then switched to 50 mM ammonium acetate, pH 4, to elute the streptavidin. A typical elution profile is shown in Fig. 4. The streptavidin-containing fractions were pooled, lyophilized, and stored at −20°C. Fig. 5 shows a typical spectrum of streptavidin before and after treatment with acetic acid followed by filtration through a G-25 Sephadex column. This acetic acid treatment enables one to more accurately determine the number of binding sites per molecule of biotin since the protein absorbance appears in a more pure form at 280 nm.

3. Gel electrophoresis: A minigel of 11% acrylamide was polymerized with ammonium persulfate and prepared with 0.1% SDS Tris HCl, pH 6.8 in the stacking gel and pH 8.85 in the running gel. Samples of Bethesda Research Laboratories streptavidin, purified streptavidin produced by *S. avidinii* as described, and concentrated fermentation supernatants were boiled in SDS-Tris-mercaptoethanol before application. The gel was stained with Coomassie blue to visualize proteins.

RESULTS AND DISCUSSION

A. Flask media studies

Media were evaluated on the basis of streptavidin production, biotin binding sites per molecule and nonspecific residues left on the iminobiotin affinity column. Of the fermentation media tested, medium H yielded the highest level of streptavidin per ml within the 48 and 72 h incubation period, as in-

Table 2

Media influence on *S. avidinii* production of streptavidin at 24, 48, and 72 h fermentation

Medium	Fermentation time (h)	Streptavidin (mg/l)	Cell wet wt. (mg/l)	Specific productivity (mg per g wet wt.)
A	24	0.5	29.0	2.0×10^{-2}
	48	7.3	345.2	2.0×10^{-2}
	72	5.3	358.0	1.5×10^{-2}
B	24	8.4	*	
	48	20.0		
	72	34.4		
C	24	8.1	448.3	1.8×10^{-2}
	48	19.8	760.1	2.6×10^{-2}
	72	47.0	1 159.2	4.1×10^{-2}
D	24	11.9	1 536.0	8.0×10^{-3}
	48	10.7	3 593.5	3.0×10^{-3}
	72	48.3	12 579.6	4.0×10^{-3}
E	24	11.3	1 802.0	6.0×10^{-3}
	48	40.7	3 593.5	1.1×10^{-2}
	72	45.0	4 089.9	1.1×10^{-2}
F	24	2.9	596.4	5.0×10^{-3}
	48	6.3	1 903.6	3.3×10^{-3}
	72	10.3	4 089.9	2.5×10^{-3}
G	24	12.6	8 997.7	1.4×10^{-3}
	48	23.2	11 750.0	2.0×10^{-3}
	72	21.5	14 855.0	1.4×10^{-3}
H	24	19.7	6 142.7	3.2×10^{-3}
	48	46.1	7 870.0	6.0×10^{-3}
	72	53.4	12 319.0	4.3×10^{-3}
I	24	8.1	498.0	1.6×10^{-2}
	48	11.4	1 054.0	1.1×10^{-2}
	72	12.1	1 240.0	1.0×10^{-2}

* Unable to weigh cells due to $CaCO_3$ present in medium.

dicated by the tritiated biotin binding assay (Table 2). Fermentation media B, C, D, E and G also produced higher yields of streptavidin when compared to media A, F, and I. The difference between these high and low streptavidin producing media is that the latter group contained only glucose as the carbon source (See Table 1). The highest specific productivity, i.e. mg product/g wet weight of cells, was observed in medium C after 72 h incubation. Medium C contained no insolubles, whereas medium H had insolubles from the corn steep liquor. Thus Medium C was chosen as the fermentor medium, since purification would be more convenient without insoluble materials. In some instances, the wet weights of the cell mass produced during 20, 48 and 72 h of fermentation may be somewhat misleading since many of the fermentation broths contained insoluble particles to which the mycelia adhered, and therefore, were part of the pellet collected upon centrifugation.

B. Fermentor runs

Assuming four biotin sites per avidin molecule, concentrations of greater than 50 mg/l have been obtained using medium C in a 10-l Chemap fermentor. The difference in final yields between fer-

mentor runs A and B may be due in part to a time period between 24 and 36 h when a problem with temperature control resulted in a temperature decrease. It was decided to continue the fermentation run in Fermentor B an additional 24 h to see if the cells could recover and continue to produce streptavidin. Fig. 2 illustrates that cell recovery was achieved and streptavidin production continued. The amount of streptavidin recovered from Fermentor A after 45 h indicated that even after two days, reasonably good yields (38.7 mg/l) were produced.

C. Assay

The tritiated biotin binding assay as described under Materials and Methods can be used to estimate the concentration of active streptavidin at all stages of the fermentation and purification. Spikes of streptavidin into culture media before growth could be quantitated within 10% of the actual value. Therefore, no materials that significantly interfere with biotin binding are present in culture media. However, some large molecular weight components of the media may also bind biotin, but less tightly than streptavidin, and account for the lower yields of purified product.

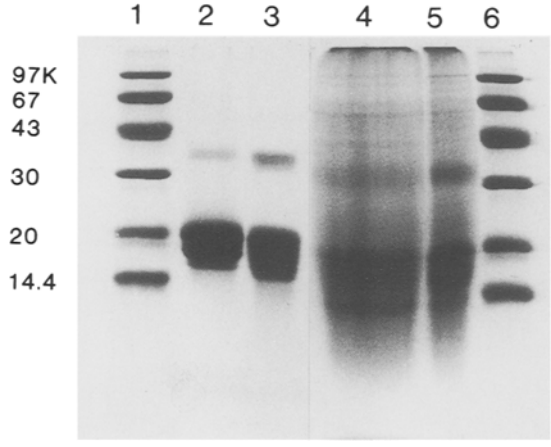

Fig. 6. SDS electrophoresis gel of streptavidin purification. Lanes 1 and 6 contain M.W. markers; 2 BRL streptavidin; 3 affinity-purified streptavidin; 4 and 5 concentrated culture supernatants.

D. Purification

Yields of streptavidin from different media varied, with an average 40% recovery of biotin binding activity as purified streptavidin. The elimination of an ammonium sulfate precipitation step, described by Hoffmann [7], prevented dark pigments from binding to the iminobiotin column and slowing the flow rate. The iminobiotin column could be regenerated by extensive washing with pH 11 buffer.

SDS-PAGE gels of the various preparations were run to confirm purity (Fig. 6). Although the major subunit of 18 kDa is diffuse, this characteristic is also observed in the fully active streptavidin from BRL in lane 2 of Fig. 6. It may represent proteolytic processing of the subunit and can also be seen in the crude supernatants. Under these reducing conditions, some dimer of the streptavidin subunit is still present both in the BRL and our purified streptavidin.

Once the streptavidin had been completley purified, the absorbency coefficient, E_{280} (1%) of 34 [3] could be used to calculate protein concentration. Biotin binding assays on the fully purified protein measured 3.5–4.0 biotins per streptavidin molecule. As the theoretical maximum for fully active streptavidin is 4.0 [2], this process yields protein of high specific activity.

CONCLUSION

From the results obtained, it is apparent that several media are well suited for the large scale production of streptavidin. The biotin-binding assay can effectively monitor streptavidin production, even in very dark media. With the simplified purification process described, the method may offer a more cost effective production of streptavidin. Although the 53 mg/l yield of streptavidin produced by *S. avidinii* under fermentor conditions (Fig. 1) was approximately half that recently reported by Cazin, et al. and Suter, et al. [11,12], our methodology has a two fold advantage; shorter fermentation time (3 vs. 8–10 days) and no extra purification steps needed for removal of cellular breakdown products that arise from prolonged fermentations. Further effi-

ciency may be gained by utilizing the more stable iminobiotin columns described by Bayer et al. [13].

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