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ENHANCEMENT BY BACTERIAL HEMOGLOBIN OF AMYLASE PRODUCTION IN RECOMBINANT *E. COLI* OCCURS UNDER CONDITIONS OF LOW O_2

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Summary

In shake flask growth, the presence of *Vitreoscilla* hemoglobin (VHb) enhanced production of recombinant α-amylase in *E. coli* by about 80% (generally confirming our previous results). Extension of these studies to growth in oxygen controlled fermentors showed that VHb afforded no advantage in α-amylase production when oxygen was not limiting, but resulted in about a 6-fold increase when oxygen was limiting. This increase was due almost entirely to secreted enzyme.

Introduction

The hemoglobin (VHb) of the obligately aerobic bacterium *Vitreoscilla* is thought to aid its host in surviving in O_2 poor environments by binding O_2 at low extracellular concentrations and buffering it at high concentrations intracellularly (Webster, 1987). Genetic engineering using the VHb gene (*vgb*) has been used in *E. coli*, other bacteria, and fungi to increase growth and production of proteins and antibiotics (Khosravi *et al.*, 1990; Khosla *et al.*, 1990; Magnolo *et al.*, 1991; DeModena *et al.*, 1993) and the biodegradation of organic compounds (Liu *et al.*, 1996). For example, we have previously shown that for shake flask growth, the presence of *vgb*/VHb in *E. coli* increased the production of recombinant α-amylase up to three-fold (Khosravi *et al.*, 1990). There is evidence that these effects are the result of more efficient use of oxygen in energy transduction, and thus greater ATP production, due to the presence of VHb (Kallio *et al.*, 1994). Because of the oxygen binding properties of VHb and its induction under hypoxic conditions (Webster, 1987), it is thought that these advantages may be enhanced at low oxygen concentrations, and there is evidence to support this idea (Khosla *et al.*, 1990; Magnolo *et al.*, 1991; DeModena *et al.*, 1993). To investigate this latter phenomenon further, we have extended our initial results in the recombinant *E. coli* α-amylase system (Khosravi *et al.*, 1990) to growth in fermentors under O_2-controlled conditions. Here we report that the advantage afforded by *vgb*/VHb in this system occurs under conditions of oxygen limitation.

Experimental Methods

Construction of the two plasmids used (pMK57 and pMK79) and their transformation into *E. coli* strain JM103 to form strains MK57 and MK79, respectively, have been described previously (Khosravi

et al., 1990). Plasmid pMK57 has a 3.0 kb fragment containing the *Bacillus stearothermophilus* α-amylase gene (Nakajima *et al.*, 1985) cloned into plasmid vector pUC8 (Messing, 1983); pMK79 was derived from pMK57 by inserting, into the latter, a 2.3 kb fragment containing the *Vitreoscilla* hemoglobin gene (*vgb*) (Dikshit and Webster, 1988; Khosla and Bailey, 1988). *Vitreoscilla* hemoglobin has been shown to be produced at high levels in strain MK79 (Khosravi *et al.*, 1990).

Culture medium was LB (ATCC medium 1065; American Type Culture Collection, 1992). As per the ATCC protocol, the pH was not adjusted; in all cases, however, it measured between 6.5-6.7 after sterilization. Strains were initially grown in a single culture each in LB, containing 100 μg/ml sodium ampicillin ("LB-Ap") and aliquots stored in 15% v/v glycerol at -70°C. For each shake flask experiment a fresh 0.5 ml aliquot of glycerol stock was cultured in 50 ml LB-Ap overnight; then 2.5 ml of this culture was added to 25 ml LB-Ap and incubated for 1 h. Following this, cells from 0.125 ml of the second culture were harvested by centrifugation, washed twice with 0.150 ml ampicillin-free LB, resuspended in 0.150 ml ampicillin-free LB and added to 50 ml of the same medium in a 250 ml Erlenmeyer flask for the experimental run (Cheah *et al.*, 1987). All incubations were done at 37°C and 125 rpm.

Fermentor experiments were set up similarly, beginning by diluting a fresh aliquot of glycerol stock 1:100 into 50 or 100 ml of LB-Ap in a shake flask and incubating overnight, followed by a one hour subculture the next day of 5 ml of overnight culture added to 100 ml LB-Ap. The entire one hour LB-Ap subculture was harvested by filtration through a 0.8 micron filter, washed on the filter using 100 ml of ampicillin-free LB, and resuspended in 100 ml ampicillin-free LB; 50 ml of this suspension was used to inoculate into 10 l of the same medium in a 10 l B. Braun Biostat™ ED ES10 fermentor. All fermentor runs were at 37°C and 350 mbar backpressure. Dissolved oxygen (DO) was measured and controlled continuously using a polarographic DO probe, and pH monitored continuously with a pH probe; both probes were from Ingold. Dissolved oxygen concentrations (measured as per cent oxygen saturation) were controlled by a combination of air flow into the headspace and agitation of the fermentors (7.5 l/min, 350 rpm for 0-3.8% DO; 15 l/min, 50-800 rpm for 20% DO). In the 20% DO runs, after a drop to about pH 6.2 at 5 hr, the pH stabilized at 7.8-8.0 after 20 hr for both strains; in the 0-3.8% DO runs, after a drop to about 6.0 at 5 hr, the pH rose steadily to 7.5-7.6 after 70 hr.

Optical densities (ODs) of cultures were measured at 600 nm versus blanks of fresh LB. Measured OD values were kept between 0.1 and 0.66 by dilution with fresh LB as necessary. Viable cell counts were determined by dilution with 10 mM sodium phosphate, 150 mM NaCl, pH 7.2, followed by plating on LB plates (to measure total (plasmid-bearing plus plasmid-free) cells). After counting, 50 colonies were selected at random from the plate titering the final time point of each run to LB-Ap plates to determine the fraction of total cells that were plasmid-bearing. For all experiments, in both shake flasks and fermentors, 100% of the viable cells contained plasmid. α-amylase was measured using an automated system (the Abbott VISION™ System); activity is reported in international units (IU). Before assaying, samples were centrifuged for 10 min at 13,500xg to separate the cells and the cell-free culture broth. α-amylase was released from the cells as described by Liu *et al.* (1992).

Results

An initial series of experiments in shake flasks was generally consistent with our previous results (Khosravi *et al.*, 1990) with strain MK79 producing considerably more intracellular α-amylase than MK57 (Figure 1). In addition, from 12 to 24 h, strain MK79 secreted much more α-amylase into the medium than did strain MK57 (Figure 1). MK57 outgrew MK79, on both viable cell (by 79%) and OD (by 17%) bases (Table 1A).

Our initial idea (Khosravi *et al.*, 1990) was that the greater α-amylase production by MK79 versus MK57 in shake flasks is due to the advantage afforded by the presence of *vgb*/VHb at the low O_2 levels occurring in the shake flasks. To test this directly, the experiments were repeated in fermentors at two DO concentrations. When DO was maintained at 20% saturation throughout the run, MK79 produced

about the same amount of secreted α-amylase and, depending on the time, about the same or even less intracellular α-amylase than MK57 (Figures 2A,C,E). MK79 was also somewhat outgrown by MK57 under these conditions (Table 1B). When the fermentor runs were repeated at DO of 0 to 3.8% saturation, MK79 produced much more total α-amylase than MK57, due mostly to its greatly increased extracellular levels of the enzyme, particularly at later times in culture; MK79 also produced, on average, somewhat higher levels of intracellular α-amylase than MK57 (Figures 2B,D,F). At 0 to 3.8% DO both strains grew much less than at 20% DO (although on an OD basis MK79 grew as well as MK57) (Tables 1B,C). Interestingly, at 0-3.8% DO MK57 produced much less α-amylase than it did at 20% DO, but this was not true for MK79.

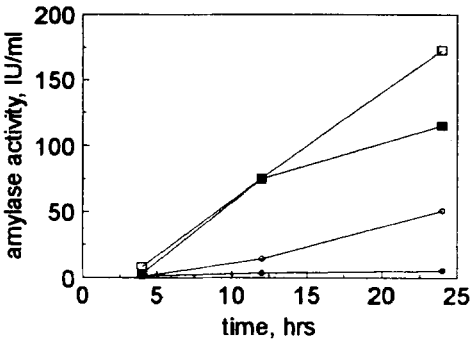
Table 1. Growth parameters for MK57 and MK79 cultured under three different conditions. (A) Viable cells/ml and OD at 600 nm plus or minus (+/-) standard errors of the mean at 24 h for shake flask cultures. Numbers in parentheses are number of individual trials in each average. (B) Maximum viable cells and OD at 600 nm for two individual fermentor runs at 20% DO. Maximum OD and viable cells occurred at 12 or 24 h. (C) Maximum viable cells and OD at 600 nm for two individual fermentor runs at 0 to 3.8% DO. Maximum viable cells occurred at 24 or 48 h for MK57 and 7.5 or 12 h for MK79. Maximum OD occurred at 70 h for both strains. In (A) the runs were 24 h; in (B) and (C) they were 70 h. Run numbers in (B) and (C) correspond to those in Figure 2.

A. Shake Flask			
Parameter		MK57	MK79
Viable cells/ml ($x10^9$)		3.4 +/- 0.7 (3)	1.9 +/- 0.3 (3)
OD		4.55 +/- 0.04 (5)	3.88 +/- 0.12 (5)

B. 20% DO			
Parameter		MK57	MK79
Maximum viable	(1)	7.2	7.3
cells/ml ($x10^9$)	(2)	8.4	5.6
Maximum OD	(1)	6.11	5.71
	(2)	6.17	5.79

C. 0-3.8% DO			
Parameter		MK57	MK79
Maximum viable	(1)	4.1	1.1
cells/ml ($x10^9$)	(2)	1.3	1.1
Maximum OD	(1)	2.40	2.50
	(2)	2.22	2.04

Figure 1. (right) Amylase production in shake flasks. ■, MK57, cellular levels; □, MK79, cellular levels; ●, MK57, secreted levels; ○, MK79, secreted levels. Cellular levels are averages of 4 or 5 individual flasks; secreted levels are averages of 2 flasks.



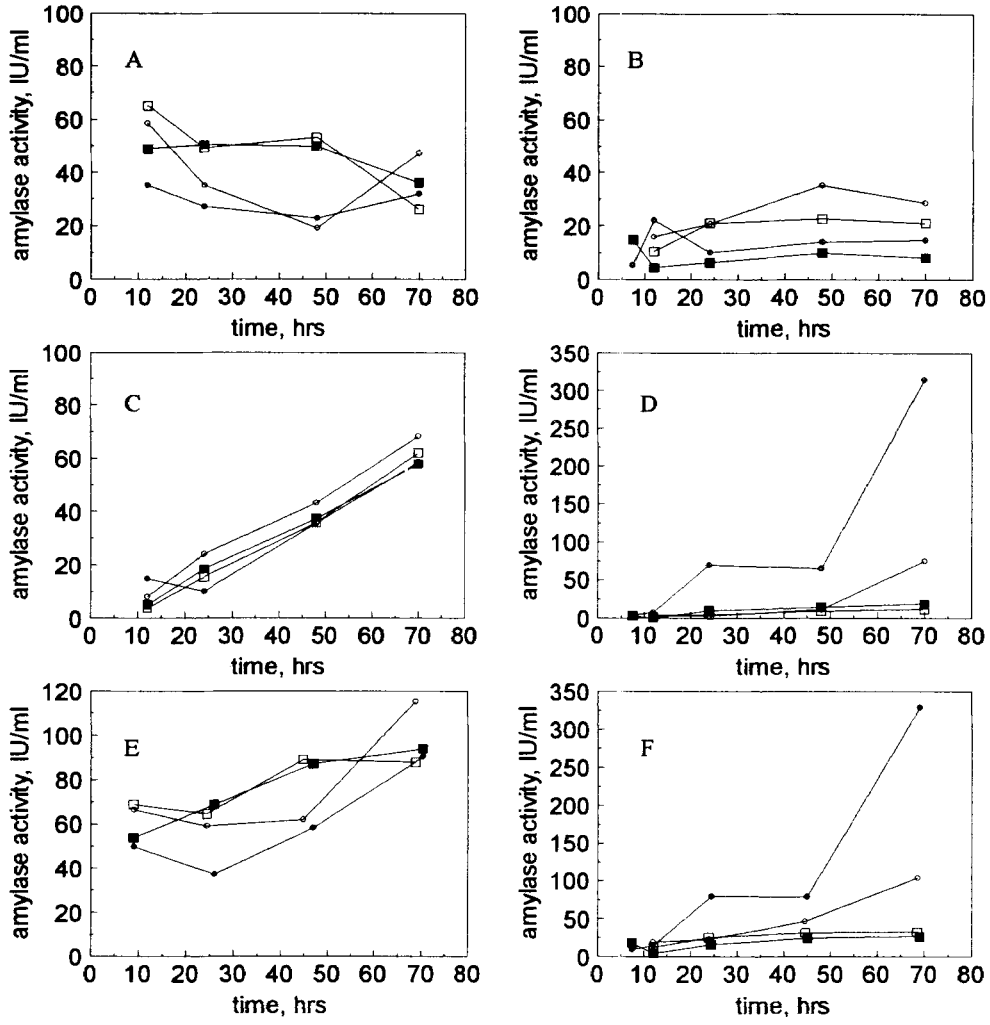


Figure 2. Amylase production in fermentors. (A) 20% DO, cellular levels; (B) 0-3.8% DO, cellular levels; (C) 20% DO, secreted levels; (D) 0-3.8% DO, secreted levels; (E) 20% DO, total (cellular plus secreted) levels; (F) 0-3.8% DO, total (cellular plus secreted) levels. ■, MK57, run 1; □, MK57, run 2; ●, MK79, run 1; ○, MK79, run 2.

Discussion

In our previous experiments comparing MK57 and MK79 shake flask growth, MK79 reached a maximum viable cell count which was 40% greater than that of MK57 (Khosravi *et al.*, 1990); OD comparisons were not made in the previous work. The reasons for the opposite trend (both viable cells and OD) in both shake flasks and fermentors in the present study are not known, but in at least one other case, a *vgb*-bearing strain has been reported to have poorer growth than a matched *vgb*-minus strain (Kallio *et al.*, 1994). In addition, our earlier experiments with strain MK79 in shake flasks showed that almost none of the α-amylase produced was extracellular (Liu *et al.*, 1992). In the prior work, however, samples were taken early in stationary phase (8 hr), and in this study the increase in α-amylase secretion in shake flasks was seen from 12 hr to 24 hr. Although both strains secreted a substantial fraction of their total α-amylase into the medium when grown under either high or low aeration in fermentors, the fraction of α-amylase secreted by MK79 under low aeration conditions was extremely high. This may be related to the much higher secretion of α-amylase by MK79, compared with MK57, in shake flask experiments, in which oxygen would be expected to be low, similar to the low aeration fermentor conditions.

The extension of our shake flask experiments to DO-controlled fermentors allowed a direct test of the key hypothesis of this study, that the increase in α-amylase production in the *vgb*-bearing strain occurs primarily at low O_2 concentrations, at which VHb should be induced to enhance O_2 uptake and utilization. This is in fact what was observed.

At 20% DO VHb should not be induced (Webster, 1987), and the two strains were similar in their α-amylase production. At 0 to 3.8% DO, VHb was probably maximally induced (Webster, 1987) and MK79 produced much more α-amylase than MK57. Under the latter conditions the advantage of MK79 was about seven-fold (on the average) on a per ml of culture volume basis. This is about twice the advantage that we observed previously for MK79 in shake flasks (Khosravi *et al.*, 1990) and is generally much greater than the VHb-related enhancement seen for other proteins produced in *E. coli* when grown under conditions of oxygen limitation (Khosla *et al.*, 1990). It is similar to the enhancement by VHb of production of two antibiotics in microorganisms grown under oxygen-limited conditions (Magnolo *et al.*, 1991; DeModena *et al.*, 1993). In any case, our data are consistent with the idea that the presence of VHb can supply O_2 more efficiently to the terminal oxidases of the electron transport chain, particularly at low DO, resulting in greater ATP production and thus greater production of valuable products, in this case, recombinant protein.

Acknowledgements

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