

Production of an active recombinant *Aspin* antigen in *Escherichia coli* for identifying animals resistant to nematode infection

Daniel Manderson^a, Robert Dempster^b, Yusuf Chisti^{a,*}

^a Institute of Technology and Engineering, Massey University, Private Bag 11 222, Palmerston North, New Zealand

^b AgResearch Ltd., Wallaceville Animal Research Center, Upper Hutt, New Zealand

Accepted 4 March 2005

Abstract

Production of recombinant *Aspin*, a aspartyl protease inhibitor homologue produced by the parasitic nematode *Trichostrongylus colubriformis*, in *Escherichia coli* is reported. Culture conditions were investigated for maximizing the production of *Aspin* in soluble bioactive form as opposed to inclusion body. High growth and expression rates caused preferential production of inclusion bodies. In fed-batch fermentations, controlling expression at low values by decreasing the bioreactor temperature, dissolved oxygen level and concentration of nutrients, all proved effective in enhancing the production of soluble *Aspin*. A high volumetric titre of 220 mg/L *Aspin* was attained in batch fermentations induced with 2 g/L L-arabinose with the postinduction temperature reduced to 25 °C from 37 °C. The pH and dissolved oxygen levels were not controlled, as acidic final pH values and low dissolved oxygen levels favored production of soluble *Aspin*.
© 2005 Elsevier Inc. All rights reserved.

Keywords: *Trichostrongylus colubriformis*; *Escherichia coli*; Nematode infection; *Aspin*; Diagnostic immunoassay

1. Introduction

Intestinal nematode infections cause major economic losses in farm animals. Infections reduce weight gain, reproductive performance and wool production. *Trichostrongylus colubriformis* (“bankrupt worm”) is a nematode parasite that infects the small intestine of sheep and goats. The parasite has significant economic impact on sheep farming in New Zealand. Currently the only effective means of controlling *T. colubriformis* is through regular use of anthelmintics. Sheep can develop immunity to *T. colubriformis* as a result of repeated natural infection [1]. Elevated immunoglobulin E (IgE) levels in sheep have been associated with protective immunity to nematode infection. Surface antigens of *T. colubriformis* have been shown to induce immunity against several other related nematodes [2].

Aspin, or Tco-API-1 [3,1], is an aspartyl protease inhibitor homologue produced by *T. colubriformis*. High titres of IgE antibodies against *Aspin* are found in lambs that have developed an effective immunity to gastrointestinal parasites [3]. Potentially, *Aspin* can be used in a simple in vitro immunodiagnostic

test to identify animals that resist infection. Previous attempts to express *Aspin* in *Escherichia coli* at Wallaceville Animal Research Center (Upper Hutt, New Zealand) have resulted in the protein almost exclusively being produced as inclusion bodies. Although a procedure for the solubilization of *Aspin* has been developed, only low activities are recovered. Therefore, this work investigated the preferential production of recombinant *Aspin* as active soluble protein in *E. coli*.

E. coli is commonly used to produce recombinant proteins because it can be grown rapidly to high densities on inexpensive media and its genetics are well understood. Unfortunately eukaryotic proteins expressed in *E. coli* commonly form insoluble inclusion bodies made up of inactive protein [4–6]. Recovery of the biologically active protein from the inclusion bodies requires solubilization and refolding that greatly add to the cost of production [7]. For some proteins, little activity is recoverable [8,38]. For this reason there is substantial interest in attempting to express the protein in a biologically active soluble form [9–12].

Culture growth rate has been shown to be important in determining the solubility of the expressed proteins [9,10,13]. At high growth rates protein expression can overwhelm the machinery required for correct folding leading to accumulation of misfolded insoluble protein [10,7]. Expression rate can be reduced,

* Corresponding author. Tel.: +64 6 350 5934; fax: +64 6 350 5604.
E-mail address: Y.Chisti@massey.ac.nz (Y. Chisti).

for example, by a lowering of the recombinant culture temperature [14–16]. Other methods of reducing inclusion body formation include using early induction of expression [17,15], nutrient and oxygen restriction to restrict the growth rate [18,19], and direct reduction of the expression rate by not fully derepressing the recombinant protein promoter [10,17].

2. Materials and methods

2.1. Strain and plasmid

Aspin was expressed in *E. coli* Rosetta-gamiTM B(DE3) (Novagen, Germany), genotype F⁻ *optM hsdSB* (r_B⁻ m_B⁻) *gal dcm lacY1 aphC gor522::Tn10(Tc^R)trxB::kan* (DE3) pRARE (Cm^R). The Rosetta-gamiTM strain has a special mutation that allows the formation of disulfide bonds [20]. All seed cultures were grown in the presence of either 34 µg/mL chloramphenicol, 15 µg/mL kanamycin or 12.5 µg/mL tetracycline. Production seeds were stored in Luria Broth with 20% glycerol at –80 °C in the presence of all three antibiotics. The *Aspin* AY2-4 expression vector kindly provided by Dr. David Maass (AgResearch Ltd, Upper Hutt, New Zealand) places the coding sequence of *Aspin* [1,3] under the control of the *araBAD* operon which is repressed in the absence of L-arabinose [21]. *Aspin* was fused to an E-tag and hexahistidine sequence to aid purification and identification [1].

2.2. Environmental screening trials

A Plackett–Burman experimental design was used to test seven environmental variables or factors for their effect on soluble *Aspin* production. Plackett–Burman is a partial factorial experimental design that is commonly used to identify the most significant variables when large numbers of experiments are prohibitive. The design requires one more experiment than the number of tested variables. Results of trials can be assessed in a simple spreadsheet using a *F*-test to determine the significance of each factor, but no information is gained on possible interaction between factors. The seven environmental factors tested included the following: culture temperature, pH, dissolved oxygen concentration, concentration of yeast extract, concentration of phosphate salts, induction time, and the concentration of L-arabinose inducer. Screening experiments were carried out in shake flasks (250 mL). Thus, 1 mL of inoculum culture was added to each of 16 flasks containing 50 mL of Terrific broth and 15 µg/mL kanamycin.

All cultures were grown in duplicate for 16 h at 180 rpm. The specific conditions used and the results are summarized in Table 1. After 16 h cell density, total protein, recombinant protein content and specific activity were measured, as detailed in the following sections.

2.3. Inducer concentration and postinduction temperature trials

The effect of varying the L-arabinose inducer concentration and postinduction temperature were assessed in shake flasks. Overnight seed (1 mL) was added to each 250 mL shake flask containing 25 mL of Terrific broth and 15 µg/mL of kanamycin. All cultures were grown at 37 °C, 180 rpm, for 8 h. For the postinduction temperature trial, the flasks temperatures were adjusted after growth to 10, 16, 19, 23, 28, and 37 °C before 2 g/L of L-arabinose was added to each flask. For the inducer concentration trial all cultures were reduced to 30 °C postinduction and induced with 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 7.5, and 10.0 g/L of L-arabinose. All experiments were performed in duplicate. Total protein, recombinant protein and final cell density were measured after 12 h, as detailed in the following sections.

2.4. Fermenter trials

All bioreactor culture trials were performed in 3.3 L roundbottomed stirred fermenter (BioFlo 3000, New Brunswick Scientific, Edison, NJ, USA) of 130 mm vessel diameter. The fermenter was fitted with pH and dissolved oxygen sensors (Mettler Toledo, OH, USA). Temperature was controlled at 37 °C via a water-filled stainless steel base. Agitation was provided by two centrally mounted six-bladed Rushton turbines spaced 80 mm apart with the lowermost impeller positioned 70 mm above the base of the vessel. Aeration occurred through a perforated pipe sparger ring. Dissolved oxygen (DO) was controlled at 30% of air saturation by using a sequential cascade of agitation between 50 and 800 rpm and aeration between 2 and 10 L/min with pure oxygen blended into the sparged air at high cell densities. The pH was controlled at 6.8 using 10% phosphoric acid and 5 M sodium hydroxide. Antifoam 289 (Sigma, St. Louis, MO, USA) was added automatically to control the foaming. The foam was sensed using a conductivity probe mounted 5 cm above the culture level. Unless stated otherwise, the initial medium volume was 1.4 L. Fermentations were inoculated with 100 mL of culture that had been grown for 16 h in a 2 L shake flask (37 °C and 180 rpm) to an optical density (595 nm) of approximately 1.5.

The feed medium of the fed-batch fermentations contained per liter: 315 g glycerol and 315 g yeast extract. Reagents and chemicals were purchased from Sigma (St. Louis, MO, USA), BioRad (Hercules, CA, USA), Merck (Darmstadt,

Table 1
Plackett–Burman trial conditions and results

Experiment	Variable ^a							
	Temperature	Inducer concentration	Induction time	Yeast extract concentration	P-salts concentration	pH	Dummy	<i>Aspin</i> (mg/mL)
1	L	L	L	H	H	L	L	0.349
2	H	L	L	L	L	L	H	0.244
3	L	H	L	L	H	H	H	0.738
4	H	H	L	H	L	H	L	0.372
5	L	L	H	H	L	H	H	0.469
6	H	L	H	L	H	H	L	0.434
7	L	H	H	L	L	L	L	0.691
8	H	H	H	H	H	L	H	0.259
∑(H)	1.309	2.060	1.854	1.449	1.781	2.013	1.711	
∑(L)	2.247	1.497	1.703	2.107	1.776	1.543	1.846	
Difference	–0.938	0.563	0.151	–0.658	0.006	0.470	–0.135	
Effect	–0.234	0.141	0.038	–0.164	0.001	0.117	–0.034	
Mean square	0.110	0.040	0.003	0.054	0.000	0.028	0.002	
<i>F</i> -test	48.239	17.414	1.257	23.754	0.002	12.119		
Significant	Yes	Yes	No	Yes	No	Yes		

^a The low (L) and high (H) levels were: 30 and 37 °C for temperature; 0.2 and 2 g/L for inducer concentration; 4 and 8 h for induction time; 5 and 24 g/L for yeast concentration; 0 and 60 mM for phosphate salt concentration; 6.8 and 7.2 for pH. A dummy variable was used to assess the degree of variability. A 95% confidence interval was used for the test of statistical significance.

Germany) and Difco (Sparks, MD, USA). Unless stated otherwise, fermentations used Terrific broth (TB) that contained per liter: 24 g yeast extract (Merck), 12 g soy-peptone (Merck), 4.8 g potassium di-hydrogen orthophosphate, 2.2 g dipotassium hydrogen orthophosphate and 5 g glycerol. A defined *E. coli* medium that has been described previously [22] was used in one of the fermentations, as identified in Section 3.

Various fed-batch trials were conducted with either pH-stat or exponential feeding. The pH-stat feeding was controlled by the automated BioCommand control software (New Brunswick Scientific). Exponential feeding began 2 h after a batch phase. The rate of feeding was calculated according to following equation [23,13]:

$$F = \frac{\mu VX}{(S_F - S)Y_{x/s}} e^{\mu t} \quad (1)$$

The specific growth rate μ in Eq. (1) was set to the desired value. In Eq. (1), t is the time (h), F the flow rate of feed at time t , V the reactor volume (L) at time t , S_F the substrate concentration in feed (g/L), S the substrate concentration in the culture broth at time t (g/L), X the cell concentration at time t (g cell dry weight/L), and $Y_{x/s}$ is the biomass yield coefficient on glucose (g dry cell weight/g).

Culture growth was monitored by measuring optical density at 595 nm using a Genesis 2C spectrophotometer (Thermo Electron Corp, New York, NY, USA). The dry cell weight (DCW, g/L) was estimated from a calibration curve that correlated experimentally measured dry weight to spectrophotometric measurements of optical density.

2.5. Protein recovery and analyses

For recovering the proteins, 20 mL of bacterial broth was centrifuged at $3000 \times g$ for 15 min. The resulting pellet was resuspended in 1.5 mL of extraction buffer (50 mM sodium phosphate, 300 mM sodium chloride, adjusted to pH 8.0 with 5N NaOH) in an eppendorf. Samples were sonicated at 50% power in 1 min bursts for 5 min using an ultrasound horn (Son-IM XL; Misonix Inc., Farmingdale, NY, USA). Cell debris and inclusion bodies were removed by centrifugation ($27,000 \times g$, 5 min). Supernatant (1 mL) was added to 50 μ L of TALON[®] metal affinity resin (Clontech Laboratories Ltd., Palo Alto, CA, USA) and agitated for ≥ 1 h. Unbound material was removed by centrifuging ($27,000 \times g$, 1 min). The resin was washed three times with the above specified extraction buffer and the bound material was eluted using 100 μ L of 100 mM Na₂EDTA.

Inclusion body protein was solubilized in 8 M urea glycine buffer. The latter contained (per liter): 484.8 g urea, 11.25 g glycine buffer, 0.37 g EDTA and had been adjusted to pH 9.0 with 10 M NaOH. Dithiothreitol (0.77 g/L) was added just prior to use. Total protein measurements used the BioRad protein assay which is based on the Bradford method [24]. Protein standards were made using bovine serum albumin (Sigma, St. Louis, MO, USA) in distilled water. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was carried out on 0.75 mm thick 15% acrylamide separating gel and 4% slackening gel, using a Mini-Protean II electrophoresis unit (BioRad, Hercules, CA, USA) [25]. Protein bands were visualized with Coomassie brilliant blue stain. Low molecular weight marker proteins were used as standards (Amersham Biosciences, Uppsala, Sweden). The quantity of Coomassie stained recombinant protein was evaluated densitometrically from SDS-PAGE gels using an GS-800 imaging densitometer (BioRad, Hercules, CA, USA) and Quantity One image quantification software (BioRad). On SDS-PAGE of affinity purified material, *Aspin* fragments occurred at 6, 22 and 28 kDa.

For *Aspin* quantification by ELISA [1], all samples were diluted to 1 μ g/mL total protein with PBS (8 g NaCl, 0.2 g KH₂PO₄·12H₂O, 0.2 g KCl per liter) and coated on a 96 well ELISA plate. After incubation (2 h, 37 °C) unbound material was poured off and the plate was washed three times with washing buffer (0.05% Tween-20 in PBS). Binding sites were then blocked for 5 min with blotto (5% non-fat milk powder in PBS) to prevent non-specific binding and the plates were rinsed six times in washing buffer. Purified IgE specific to *Aspin* [1] was then added to the wells and incubated overnight (37 °C). The wells were then washed and probed with monoclonal xB6/xD3 (kindly provided by Richard Shaw, AgResearch Ltd, Upper Hutt, New Zealand). A goat-anti-mouse horse radish peroxidase (HPR) conjugate (Gibco; Invitrogen Corporation, Carlsbad, CA, USA) was added. The color was developed using 3,3',5,5'-tetramethylbenzidine (TMB) substrate. The absorbance was measured at 630 and 450 nm with a microtitre plate reader (MR5000; Dynatech Laboratories Inc., Chantilly, VA, USA). The measured absorbance was compared with standard curve prepared using a sample of known activity [1]. All three *Aspin* fragments were recognized by the ELISA used.

3. Results and discussion

3.1. Environmental screening trials

A Plackett–Burman trial was used to test seven environmental variables (i.e. culture temperature, pH, dissolved oxygen concentration, concentration of yeast extract, concentration of phosphate salts, induction time, and the concentration of L-arabinose inducer) for their effect on soluble *Aspin* production. The specific conditions used and the results are summarized in Table 1. The *F*-test significance of each variable on volumetric production, cell specific production and activity of *Aspin*, are shown in Table 2.

Reducing the culture temperature from 37 to 30 °C had the greatest effect on the biomass specific production of soluble *Aspin* and its specific activity (Table 2). This effect of postinduction temperature on expression of active soluble protein has been reported for many other proteins [9,21,26,15,12]. A lowering of temperature reduces growth and synthesis rates, allowing more time for the folding mechanisms to correctly fold the protein into active soluble form [27]. In view of its high significance (Table 2), the postinduction temperature was selected as a variable for further investigation.

The concentration of yeast extract in the growth medium had a strong negative effect on the soluble *Aspin* titre and the biomass specific activity of the recombinant protein (Table 2). This was likely because yeast extract provided a readily accessible source of amino acids and trace metals [28,29], to enhance the cell growth rate. Cultures grown in media rich in yeast extract are known to experience high growth rates [11]. Increased growth associated with a high rate of total protein synthesis usu-

Table 2

F-test significance of various environmental factors on volumetric and biomass specific expression of *Aspin* and the activity of the recombinant *Aspin* produced

Response	Significant environmental conditions ^a					
	Temperature	Inducer concentration	Induction time	Yeast concentration	P-salt concentration	pH
<i>Aspin</i> (μ g/mL)	48.2 (–)	17.4 (+)	1.3 (+)	23.8 (–)	0.0 (+)	12.1 (–)
<i>Aspin</i> (μ g/mg DCW)	135.0 (–)	47.1 (+)	1.8 (+)	65.1 (–)	0.0 (+)	32.1 (–)
Activity (U/mg <i>Aspin</i>)	22.3 (–)	10.8 (+)	1.4 (+)	11.5 (–)	0.9 (–)	0.4 (–)

^a Values greater than 4.8 indicate statistical significance at 95% confidence level. The signs in parentheses indicate whether changing from the low to the high value had a negative or positive effect.

ally causes increased protein aggregation. Some sources have reported that media rich in yeast extract elevate expression of soluble proteins in high-cell-density cultures, but these reports have focused on volumetric titres and not the biomass specific titres [11]. In rich media, volumetric concentration of the desired protein may be high simply because of the high cell density, even though the cell specific production of the protein may be low. In the range investigated in this work, the concentration of yeast extract had a marginal positive effect on the final biomass level only at the highest tested concentration of the yeast extract.

The concentration of the inducer (L-arabinose) had a strong effect on both the amount of soluble *Aspin* produced and its specific activity (Table 2). Increasing the L-arabinose concentration from 0.2 to 2 g/L led to an increase in the titre of soluble *Aspin* and the total inclusion body protein. This effect was further studied and is discussed in Section 3.2.

The timing of induction had no statistically significant effect on either the titre of *Aspin* or its specific activity (Table 2). Early induction is known to increase soluble protein production of some recombinant proteins by limiting the culture growth rate [15,30,39]. For example, Lim and Jung [30] found that induction in early logarithmic phase gave a five-fold improvement in soluble interferon- α production compared to when the culture was induced in late logarithmic phase. The effect of induction timing appears to depend on the specific case. Thus, other studies have reported higher volumetric levels of protein expression when induced in the late exponential growth phase [31,32]. Early induction in the present study was observed to have little overall effect on the growth rate of cells and all *Aspin* cultures achieved similar final cell densities.

Media supplementation with inorganic phosphate has been reported to increase cell growth and recombinant protein production [19,22]. Ryan et al. [19] tested various levels of phosphate and found that both cell growth rate and β -lactamase production were increased when culture phosphate levels were supplemented to 128 mM. In the present study, the basal medium contained sufficient phosphate and further supplementation had no effect on the production or activity of *Aspin* (Table 2). (Terrific broth is rich in peptone and yeast extract that contain approximately 10% w/w phosphate.)

The solubility of recombinant proteins expressed in *E. coli* has been reported to be affected by pH [8]. Reducing the pH from 7.2 to 6.8 led to an increase in both volumetric and specific titres of soluble *Aspin* (Table 2). This is at odds with Strandberg and Enfors [8] who found that amount of recombinant β -galactosidase expressed as inclusion bodies increased with decreasing pH. The pH range for optimal folding of different proteins appears to be different [19] and any generalization concerning the effect of pH on folding is difficult because of the complex interactions among sequences of amino acid residues.

3.2. Inducer concentration effects

The effect of varying L-arabinose inducer concentration on total volumetric protein production is shown in Fig. 1a. Maximum protein production occurred at inducer concentration of 2.5 g/L. Higher levels of arabinose reduced protein expression.

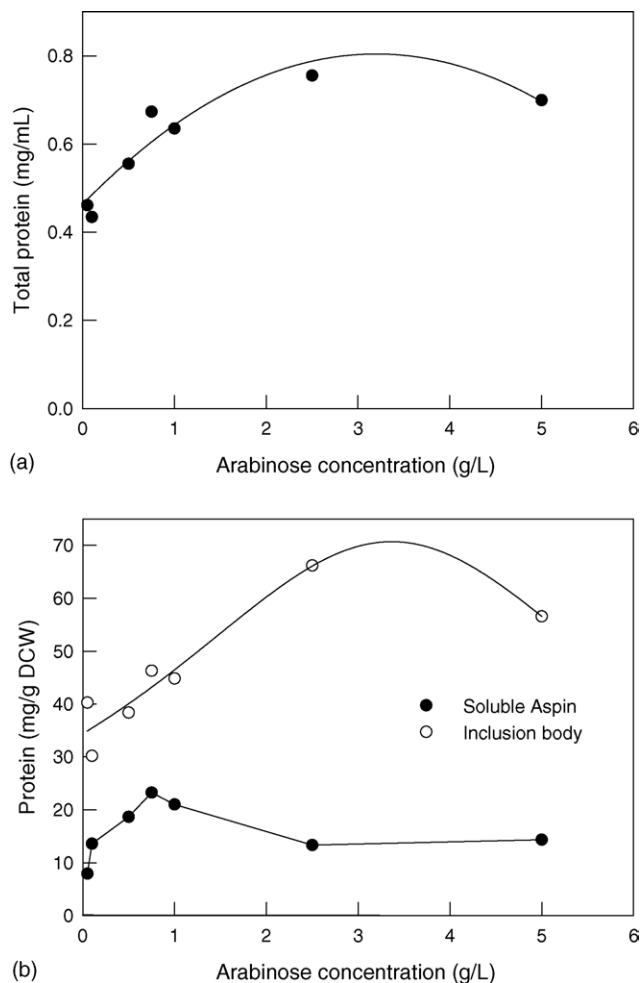


Fig. 1. (a) Total production of *Aspin* with various concentrations of inducing agent; (b) effect of inducing agent concentration on soluble *Aspin* and insoluble protein production.

Concentrations of L-arabinose of between 0.1 and 2.0 g/L have been used to induce production of various other recombinant proteins [21,27,17,33]. Choi et al. [33] used 1 g/L of L-arabinose to induce the production of human granulocyte colony stimulating factor (hG-CSF) to a level of 6–9 g/L in a high-cell-density fermentation of an *E. coli* strain that did not metabolize L-arabinose. The high concentration requirement for L-arabinose by the *Aspin* producing strain of our work is likely attributed to its being able to metabolize L-arabinose.

For otherwise identical conditions, the inducer concentration affected titres of soluble and insoluble *Aspin* to different degrees (Fig. 1b). Increasing the L-arabinose concentration from 0.05 to 0.75 g/L increased cell specific production of both soluble and insoluble *Aspin*. Above a concentration of 0.75 g/L, the increasing inducer concentration caused a slight decline in the cell specific concentration of the soluble protein, but the concentration of the inclusion body *Aspin* continued to increase until the optimal inducer concentration of 2.5 g/L.

This behavior was associated with the nature of the *araBAD* promoter. The *araBAD* is a strongly regulated promoter and its expression level is modulated by the concentration of arabinose [21]. As the concentration of the inducer increased, the protein

expression level increased, but above a inducer concentration of 0.75 g/L the cell's protein folding machinery could no longer process the recombinant protein into a correctly folded form and the excess protein ended up as inclusion body. The restriction of recombinant expression rate through partial induction has been shown to favor the formation of soluble protein [10,34,17]. Lim et al. [17] observed a halving in the interferon- α production when the concentration of L-arabinose was reduced from 2 to 0.4 g/L; however, the fraction of interferon- α produced in the soluble form increased from less than 5 to 80%.

The reduced inclusion body production seen in Fig. 1b for a inducer concentration of 5 g/L was likely a consequence of hyper-production. High concentrations of arabinose are known to cause hyper-production of recombinant protein (>30% of total cellular protein), which can lead to destruction of ribosomes, production of heat shock proteins and cell death [21]. The *araBAD* promoter has been previously shown to be strongly regulated with a low level of basal expression [21,27]. In the absence of L-arabinose, 0.053 g/g DCW of soluble *Aspin* was produced (data not shown). For systems in which the reduction of basal expression is important, the addition of glucose to the growth media causes strong repression of the *araC* gene which regulates the *araBAD* promoter [27].

3.3. Postinduction temperature

The variations of the biomass specific concentrations of soluble and inclusion body *Aspin*, for various postinduction temperatures are shown in Fig. 2a. For both forms of *Aspin*, the concentration in the cells increased as the postinduction temperature was raised from 10 to 28 °C. This was because the rate of protein synthesis increased with increasing temperature. In Fig. 2a, the concentration of soluble *Aspin* declined as the postinduction temperature was further raised from 28 °C to the optimal growth temperature of 37 °C. This was because even though the total rate of protein synthesis increased or levelled off, the high rate of synthesis overwhelmed the protein folding machinery. This is seen clearly in Fig. 2b, where the cell specific production of total recombinant *Aspin* increases until about 30 °C, but the soluble *Aspin* concentration in the cells peaks at a significantly lower temperature.

For recombinant proteins that have a tendency to form insoluble aggregates in *E. coli*, decreasing the culture temperature has been shown to reduce protein aggregation [10,11,7,16,12]. Reducing the postinduction temperature from 37 to 28 °C led to a doubling of the biomass specific titre of soluble *Aspin*. This is consistent with Schein and Noteborn [9] who tested a number of *E. coli* strains and plasmid constructs and found that the soluble fraction of the recombinant protein could be increased by using a lower culture temperature in the range of 20–30 °C. Lowering the temperature apparently slows protein biosynthesis more than the folding mechanisms, so that accumulation of protein as inclusion bodies is reduced [9,10,27]. Lower cultivation temperatures are also claimed to reduce stress responses during induction and product degradation by proteases [14].

An experiment was run with a postinduction temperature of 42 °C (data not shown). As expected, the production of *Aspin*

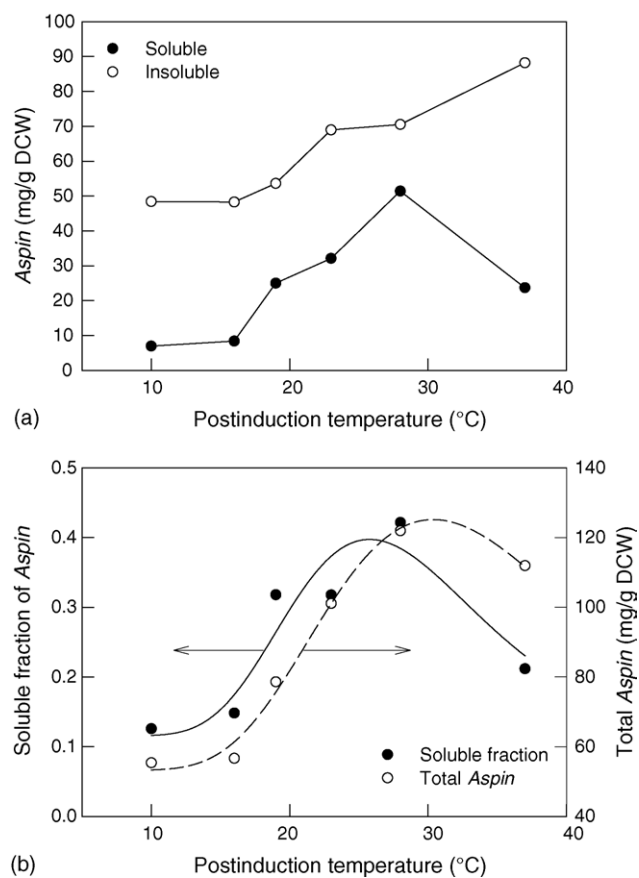


Fig. 2. (a) Effect of postinduction temperature on the production of specific soluble *Aspin* and insoluble protein. Inducer concentration was 2 g/L. (b) Soluble fraction of *Aspin* and total (soluble and insoluble) biomass specific *Aspin* produced at various postinduction temperatures.

was less than when the culture was induced at 37 °C; however, the magnitude of the effect was unexpected. At 42 °C, 0.034 g/g DCW of soluble *Aspin* was produced, or less than observed in the absence of the inducing agent. The final cell-density and amount of inclusion protein were also the lowest compared to the other trials. Within limits, up-shifting the culture temperature is known to increase the plasmid copy number and rate of recombinant protein synthesis and this is known to favor increased inclusion body formation [16].

3.4. Fermenter trials

Further assessments of recombinant *Aspin* production were carried out in the controlled environment of a bioreactor to facilitate development of a commercial production process. Fermentations were performed in fed-batch mode after an initial batch phase. Fed batch feeding comprised pH-stat feeding in one fermentation and exponential feeding in the others. The results are shown in Table 3. The pH-stat fed fermentation attained a final cell density of 21.9 g DCW/L after 24 h (Table 3); however, both the volumetric and cell specific titres of soluble *Aspin* were quite low even though the production of the inclusion body protein was comparable to some of the cultures fed exponentially.

Table 3
Summary of growth conditions and production characteristics of *Aspin* fermentations

Feeding method ^a	Fermentation conditions					
	Postinduction temperature (°C)	Postinduction growth rate (h ⁻¹)	Final cell density (g/L)	Inclusion body protein (g/L)	Soluble <i>Aspin</i> (g/L)	Specific soluble <i>Aspin</i> (g/g DCW)
None (shake flask)	28	^b	0.91	0.448	0.239	0.259
pH-stat	28	0.052	21.9	2.585	0.008	0.0004
Exponential	25	0.037	22.6	2.721	0.085	0.004
Exponential	25	0.046	14.8	1.982	0.023	0.002
Exponential	25	0.033	13.1	^c	0.027	0.002
None	23	0.019	0.96	0.416	0.222	0.231
None	19	0.030	0.92	0.322	0.084	0.091

^a Fermentation 5 used a defined *E. coli* medium [22]; all other fermentations used Terrific broth. Fermentations 6 and 7 used a constant aeration of rate of 0.8 L/min, agitation speed of 350 rpm, without control of dissolved oxygen and pH.

^b Postinduction growth rate not measured.

^c No inclusion body pellet recovered.

The pH-stat culture had the highest postinduction growth rate compared to the other fermentations and this was the likely cause of the low titre of soluble *Aspin*. As noted earlier, high postinduction growth rates are known to cause recombinant eukaryotic proteins produced in *E. coli* to aggregate and form inclusion bodies [35,26,10,11,32,12]. Indeed, proteins endogenous to *E. coli* can accumulate as inclusion bodies if expressed at a high rate [35], suggesting that inclusion body formation is a consequence of high expression rate regardless of the protein being expressed. A total of 2.6 g/L of inclusion body protein was produced with a specific titre of 118 mg/g DCW, but the content of *Aspin* could not be quantified because of the presence of a large number of impurities.

Subsequent fermentations used various strategies to control the specific growth rate at lower values than attained in the pH-stat culture (Table 3), to enhance production of soluble *Aspin*. The strategies used included a lowering of the postinduction temperature to 25 °C, use of a low feed rate in exponential feeding and control of the dissolved oxygen supply. Similar approaches have been described by others [32,36,39].

Thus, in fermentations 3–5 in Table 3, the postinduction temperature was reduced to 25 °C with the postinduction growth rates controlled at 0.037, 0.046 and 0.033 h⁻¹, respectively. Fermentation 3 gave a final (24 h) biomass concentration that was comparable to the faster grown pH-stat fermentation (i.e. fermentation 2). The total inclusion body proteins levels in the two cultures were quite comparable, but the slower grown fermentation produced nearly 10 times as much soluble *Aspin* as the faster grown pH-stat culture. Fermentation 5 (Table 3) also had a low postinduction growth rate, but produced a low level of biomass and no recoverable inclusion body. Significantly more soluble *Aspin* was produced than in the pH-stat mode. Fermentation 5 was conducted using limited nutrient levels of a defined *E. coli* medium [22]. The cell specific *Aspin* production was half of that in fermentation 3. The defined medium was assessed because such media are known to reduce batch-to-batch variability of a fermentation and feeding can be better managed because of well known levels of nutrients in the medium. Compared to complex media, synthetic media are well known to reduce growth rate because the cells need to synthesize their

own amino acids and this ties up metabolic capacity [21,29]. Fermentation 5 took 20 h to reach a cell density of 10 g DCW/L at which time it was induced with 2 g/L of L-arabinose. The postinduction growth rate was accurately controlled with the actual measured rate being 0.033 h⁻¹. The culture took 28 h to attain the 13.1 g DCW/L biomass concentration. In agreement with this work, use of defined media has generally lowered the titres of recombinant proteins [31,11].

In view of these results, reducing the growth rate is clearly not the sole factor that determines recombinant protein synthesis and the specific method used to reduce growth is important. In this study, controlled feeding with a rich medium was clearly better than using a nutrient poor defined medium to control growth rate. However, despite lower titres, using a chemically defined medium in commercial processes can have important advantages [6].

Although the pH-stat and exponentially fed fermentations provided much useful information about the production of *Aspin*, they did not attain the high levels of soluble *Aspin* and cell specific *Aspin* that were attained in shake flasks (e.g. fermentation 1 in Table 3). Thus, the highest level of *Aspin* obtained in the fermenter was still less than half of that attained in shake flasks. Considering this, attempts were made to emulate the shake flask conditions in the fermenter. Thus, the aeration rate was lowered to 0.8 L/min, the agitation rate was fixed at 350 rpm, the culture was not fed and the pH was not controlled. Postinduction, the temperature was reduced to 23 °C after 8 h of growth (fermentation 6 in Table 3). The fermentation profiles of dissolved oxygen and pH variations are shown in Fig. 3. With these conditions in the fermenter, the final cell density after 24 h was only 0.910 g DCW/L and the postinduction growth rate was 0.019 h⁻¹ (fermentation 6 in Table 3). These results were comparable to those obtained in the shake flask (fermentation 1 in Table 3) and, predictably, the inclusion body concentration, the soluble amount of *Aspin* and its cell specific production compared closely with corresponding values of the shake flask. Apparently, the low growth rate caused by a low and diminishing level of dissolved oxygen (Fig. 3) reduced cell growth and contributed to occurrence of high *Aspin* titres. The lowering of the growth rate by restricted oxygen supply has previously been used to increase

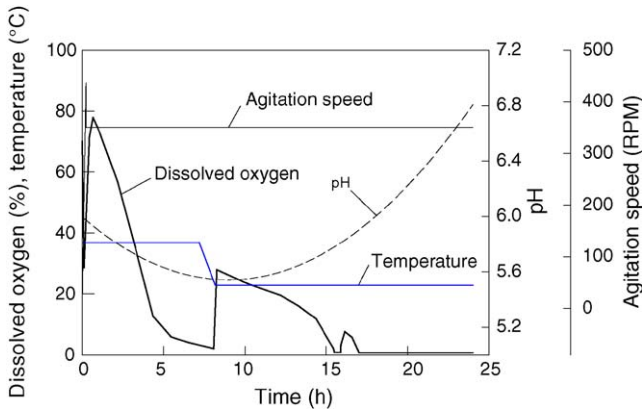


Fig. 3. Dissolved oxygen and pH profiles of fermentation 6.

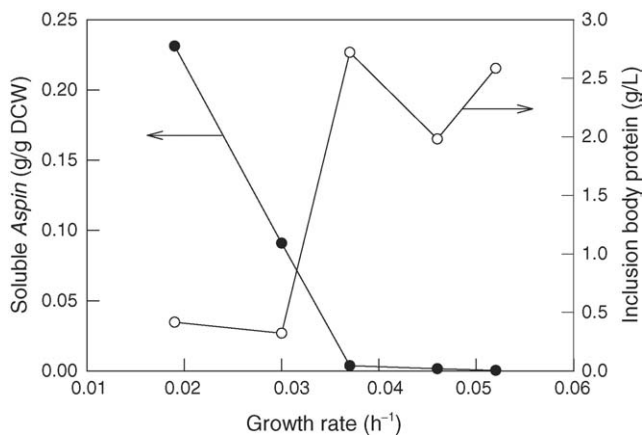


Fig. 4. Effect of postinduction growth rate on soluble *Aspin* and inclusion body production in fermenter cultures.

recombinant protein titres [18,19]. An additional contributory factor to attaining high *Aspin* titres was the decline in pH during the uncontrolled fermentation (Fig. 3). The screening experiments (Table 1) had earlier revealed an improved expression of *Aspin* at low pH. Fig. 4 plotted using data from the controlled fermentations (i.e. fermentations 2–4, 6 and 7) clearly shows that increasing postinduction growth rate reduces production of soluble *Aspin* and increases production of protein in the form of inclusion body.

4. Concluding remarks

Concentration of L-arabinose inducer affected both the total protein expressed and production of the soluble *Aspin*. L-arabinose concentration of 0.75 g/L was optimal for maximizing the production of soluble *Aspin*, but a higher concentration of 1.2 g/L was needed for maximizing total recombinant protein expression.

The production of soluble *Aspin* was found to be highly dependant on the rate of expression. At high rates of expression the cellular protein folding mechanisms were overwhelmed, leading to accumulation of protein as inclusion bodies. The following methods of reducing the growth rate proved effective in enhancing the relative amount of the soluble *Aspin*: reducing the

postinduction culture temperature, reduced feeding rate, and limitation of dissolved oxygen concentration. The titre of soluble *Aspin* doubled when the postinduction temperature was reduced from 37 to 28 °C.

Using fed-batch techniques in the fermenter allowed production of high cell densities, but any *Aspin* was produced almost exclusively in inclusion bodies. A bioreactor fermentation that emulated the conditions of shake flasks, achieved a soluble *Aspin* titre of 222 mg/L. Prior to this study all attempts to produce *Aspin* in a fermenter had produced the protein exclusively as insoluble inclusion bodies. The soluble *Aspin* titre of 222 mg/L attained in the best case bioreactor was relatively high compared to other published data. Only a few publications have reported attaining soluble recombinant eukaryote protein titres at levels of 200–500 mg/L using *E. coli* [11,37]. Generally, the titre values have ranged over 1–40 mg/L [15,12]. In view of comparatively high titres, production of soluble *Aspin* in commercial fermenters using *E. coli* is feasible and will eliminate the expensive additional processing that would be required if production occurred in the form of inclusion bodies.

References

- [1] Shaw RJ, McNeill MM, Maas DR, Hein WR, Barber TK, Wheeler M, et al. Identification and characterisation of an aspartyl protease inhibitor homologue as a major allergen of *Trichostrongylus colubriformis*. *Int J Parasitol* 2003;33:1233–43.
- [2] Harrison GBL, Pulford HD, Hein WR, Barber TK, Shaw RJ, McNeill M, et al. Immune rejection of *Trichostrongylus colubriformis* in sheep; a possible role for intestinal mucus antibody against an L3-specific surface antigen. *Parasite Immunol* 2003;25:45–53.
- [3] Shaw RJ, Gatehouse TK, McNeill MM. Serum IgE responses during primary and challenge infections of sheep with *Trichostrongylus colubriformis*. *Int J Parasitol* 1998;28:293–302.
- [4] Dempster RP, Robinson CM, Harrison GBL. Parasite vaccine development: large-scale recovery of immunogenic *Taenias ovis* fusion protein GST-45W(B/X) from *Escherichia coli* inclusion bodies. *Parasitol Res* 1996;82:291–6.
- [5] Makrides SC. Strategies for achieving high-level expression of genes in *Escherichia coli*. *Microbiol Rev* 1996;60:512–38.
- [6] Chisti Y. Strategies in downstream processing. In: Subramanian G, editor. *Bioprocess and bioprocessing: a handbook*, vol. 2. New York: Wiley-VCH; 1998. p. 3–30.
- [7] Lilie H, Schwarz E, Rudolph R. Advances in refolding of proteins produced in *E. coli*. *Curr Opin Biotechnol* 1998;9:497–501.
- [8] Strandberg L, Enfors S-O. Factors influencing inclusion body formation in the production of a fused protein in *Escherichia coli*. *Appl Environ Microbiol* 1991;57:1669–74.
- [9] Schein CH, Noteborn MHM. Formation of soluble recombinant proteins in *Escherichia coli* is favoured by lower growth temperature. *Biotechnol* 1988;6:291–4.
- [10] Georgiou G, Valax P. Expression of correctly folded proteins in *Escherichia coli*. *Curr Opin Biotechnol* 1996;7:190–7.
- [11] Zanette D, Dundon W, Soffientini A, Sottani C, Marinelli F, Akeson A, et al. Human IL-1 receptor antagonist from *Escherichia coli*: large-scale microbial growth and protein purification. *J Biotechnol* 1998;64:187–96.
- [12] MacDonald LM, Armson A, Thompson RCA, Reynoldson JA. Characterization of factors favouring the expression of soluble protozoan tubulin proteins in *Escherichia coli*. *Protein Express Pur* 2003;29:117–22.
- [13] Lee J, Lee SY, Park S. Fed-batch culture of *Escherichia coli* w by exponential feeding of sucrose as a carbon source. *Biotechnol Techniques* 1997;11:59–62.

- [14] Madurawe RD, Chase TE, Tsao EI, Bentley WE. A recombinant lipoprotein antigen against Lyme disease expressed in *E. coli*: fermentor operating strategies for improved yield. *Biotechnol Prog* 2000;16:571–6.
- [15] Eriksen NT, Kratchmarova I, Neve S, Kristiansen K, Iversen JLL. Automatic inducer addition and harvesting of recombinant *Escherichia coli* cultures based on indirect on-line estimation of biomass concentration and specific growth rate. *Biotechnol Bioeng* 2001;75:355–61.
- [16] Hoffmann F, Rinas U. Plasmid amplification in *Escherichia coli* after temperature upshift is impaired by induction of recombinant protein synthesis. *Biotechnol Lett* 2001;23:1819–25.
- [17] Lim H-K, Jung K-H, Park D-H, Chung S-I. Production characteristics of interferon- α using an L-arabinose promoter system in a high-cell-density culture. *Appl Microbiol Biotechnol* 2000;53:201–8.
- [18] Unutmaz C, Cail RG, Haggert KD. Increased α -lytic protease production from recombinant *Escherichia coli* grown with oxygen limitation. *Biotechnol Lett* 1997;19:731–4.
- [19] Ryan W, Parulekar SJ, Stark BC. Expression of β -lactamase by recombinant *Escherichia coli* strains containing plasmids of different sizes-effect of pH, phosphate, dissolved oxygen. *Biotechnol Bioeng* 1989;34:309–19.
- [20] Derman AI, Prinz WA, Belin D, Beckwith J. Mutation that allow disulfide bond formation in the cytoplasm of *Escherichia coli*. *Science* 1993;262:1744–8.
- [21] Guzman L-M, Belin D, Carson MJ, Beckwith J. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J Bacteriol* 1995;177:4121–30.
- [22] Korz DJ, Rinas U, Hellmuth K, Sanders EA, Deckwer W-D. Simple fed-batch technique for high cell density cultivation of *Escherichia coli*. *J Biotechnol* 1995;39:59–65.
- [23] Ejiofor AO, Chisti Y, Moo-Young M. Culture of *Saccharomyces cerevisiae* on hydrolysed waste cassava starch for production of baking-quality yeast. *Enzyme Microb Technol* 1996;18:519–25.
- [24] Bradford M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248–54.
- [25] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–5.
- [26] Ramirez DM, Bentley WE. Fed-batch feeding and induction policies that improve foreign protein synthesis and stability by avoiding stress responses. *Biotechnol Bioeng* 1995;47:596–608.
- [27] Chang CC, Ryu DDY, Park CS, Kim J-Y, Ogrzydziak DM. Recombinant bioprocess optimization for heterologous protein production using two-stage, cyclic fed-batch culture. *Appl Microbiol Biotechnol* 1998;49:531–7.
- [28] Kern M, Nidetzky B, Kulbe KD, Haltrich D. Effect of nitrogen sources on the levels of aldose reductase and xylitol dehydrogenase activities in the xylose-fermenting yeast *Candida tenuis*. *J Ferment Bioeng* 1998;85:196–202.
- [29] Zhang J, Greasham R. Chemically defined media for commercial fermentations. *Appl Microbiol Biotechnol* 1999;51:407–21.
- [30] Lim H-K, Jung K-H. Improvement of heterologous protein productivity by controlling postinduction specific growth rate in recombinant *Escherichia coli* under control of the P_L promoter. *Biotechnol Prog* 1998;14:548–53.
- [31] Yee L, Blanch HW. Recombinant trypsin production in high cell density fed-batch cultures of *Escherichia coli*. *Biotechnol Bioeng* 1993;41:781–90.
- [32] Akesson M, Hagander P, Axelsson J. Avoiding acetate accumulation in *Escherichia coli* cultures using feedback control of glucose feeding. *Biotechnol Bioeng* 2001;73:223–30.
- [33] Choi S-J, Park D-H, Jung K-H. Development and optimisation of two-stage cyclic fed-batch culture for hg-csf production using L-arabinose promoter of *Escherichia coli*. *Bioproc Biosys Eng* 2001;24:51–8.
- [34] Martinez A, Ramirez OT, Valle F. Effect of growth rate on the production of β -galactosidase from *Escherichia coli* in *Bacillus subtilis* using glucose-limited exponentially fedbatch cultures. *Enzyme Microb Technol* 1998;22:520–6.
- [35] Gribskov M, Burgess RR. Overexpression and purification of the sigma subunit of *Escherichia coli* RNA polymerase. *Gene* 1983;26:109–18.
- [36] Castan A, Enfors S-O. Characteristics of a DO-controlled fed-batch culture of *Escherichia coli*. *Bioproc Eng* 2000;22:509–15.
- [37] Jeong KJ, Lee YL. Excretion of human β -endorphine into culture medium by using outer membrane protein f as a fusion partner in recombinant *Escherichia coli*. *Appl Environ Microbiol* 2002;68:4979–85.
- [38] Fischer B, Sumner I, Goodenough P. Isolation, renaturation, and formation of disulfide bonds of eukaryotic proteins expressed in *Escherichia coli* as inclusion bodies. *Biotechnol Bioeng* 1993;41:3–13.
- [39] DeLisa MP, Chae HJ, Weigand WA, Valdes JJ, Rao G, Bentley WE. Generic model control of induced protein expression in high cell density cultivation of *Escherichia coli* using on-line GFP-fusion monitoring. *Bioproc Biosys Eng* 2001;24:83–91.