APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1991, p. 1669–1674
0099-2240/91/061669-06$02.00/0

Vol. 57, No. 6

Factors Influencing Inclusion Body Formation in the Production of a Fused Protein in *Escherichia coli*

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Received 14 January 1991/Accepted 27 March 1991

Different parameters that influenced the formation of inclusion bodies in *Escherichia coli* during production of a fused protein consisting of protein A from *Staphylococcus aureus* and β-galactosidase from *E. coli* were examined. The intracellular expression of the fused protein was controlled by the p_R promoter and its temperature-sensitive repressor. The induction temperature, the pH of the cultivation medium, and changes in the amino acid sequence in the linker region between protein A and β-galactosidase had a profound effect on the formation of inclusion bodies. At 42°C, inclusion bodies were formed only during the first hours after induction, and thereafter all the recombinant protein that was further produced appeared in a soluble and active state. Production at 39 and 44°C resulted in inclusion body formation throughout the production period with 15 to 20% of the produced recombinant protein appearing as inclusion bodies. Cultivating cells without control of pH caused inclusion body formation throughout the induction period, and inclusion body formation increased with decreasing pH, and at least part of the insoluble protein was formed from the pool of soluble fusion protein within the cell. Changes in the amino acid sequence in the linker region between the two parts of the fusion protein abolished inclusion body formation.

In the production of recombinant protein in microorganisms, one phenomenon that often is encountered is the formation of inclusion bodies; that is, the recombinant protein aggregates in an insoluble form in the cytoplasm or periplasm. This formation of inclusion bodies can create both opportunities and problems.

For proteins that are degraded by proteases when expressed in microorganisms, the production of the sensitive protein in the form of inclusion bodies could offer a possibility to stabilize the protein (9). For purification purposes, the formation of inclusion bodies offers both an initial purification and a concentration of the recombinant protein since inclusion bodies can, after disintegration of the cells, be separated from cell debris and soluble protein by centrifugation. However, to renaturate the aggregated protein to a native conformation usually requires several steps, including the handling of large volumes of solution (in process scale). The yields are often low (20).

The mechanism behind inclusion body formation is not well understood. Early hypotheses included solubility limitations, protein size, type of promoter, and improper disulfide formation, but comparison of literature data shows no simple relationship between these factors and the formation of inclusion bodies (16, 20, 30). It has been suggested that inclusion bodies are formed from intermediates of the folding pathway (21), and increased growth temperature for the producing bacteria is one parameter that has been reported to promote the aggregation of recombinant protein as inclusion bodies (3, 4, 31).

Here we report on inclusion body formation of a model protein which can be produced in a soluble or precipitated form, depending on the process conditions. The studied protein is a fusion protein consisting of the five immunoglobulin G-binding domains of protein A from *Staphylococcus aureus* fused to β-galactosidase from *Escherichia coli* (24). The intracellular expression in *E. coli* of the fused protein, hereafter called SpA-βgal, was placed under the control of the p_R promoter and its temperature-sensitive repressor protein. SpA-βgal is proteolytically stable in the cytoplasm (9, 11).

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MATERIALS AND METHODS

Strain and cultivation conditions. The SpA-βgal-producing strain was *E. coli* RR1 *lacZ*ΔM15 (28). pRIT1 (24) and pRIT1b (9) encode two different SpA-βgal constructs as well as ampicillin resistance. The difference between pRIT1 and pRIT1b lies within the linker region between protein A and β-galactosidase. The fusion point for the linker in protein A is amino acid 270, which is within the C-terminal immunoglobulin G-binding domain of protein A. The fusion point in β-galactosidase is amino acid 24 in pRIT1 and amino acid 7 in pRIT1b. pNF2690 (24) and pRITcI857 (23) both encode the temperature-sensitive repressor of the p_R promoter and kanamycin resistance. The *E. coli* strain was cotransformed with pRIT1 and pNF2690 or with pRIT1b and pRITcI857. pRITcI857 has replaced pNF2690 as a source for the temperature-sensitive repressor protein in our research projects, since pRITcI857 is an in house-constructed plasmid. Cultivations were performed in a 5-liter (working volume) Chemap SG7 bioreactor. The pH in the bioreactor was controlled at 7.0 with 4 M NaOH unless otherwise stated. The temperature was held at 30°C during the growth phase and then raised to temperatures between 39 and 44°C, as stated in the text, to initiate the expression of SpA-βgal. The aeration rate was held at 0.25 VVM (liters of air liter of medium^{-1} min^{-1}) until the optical density at 610 nm (OD_{610}) reached 10 and thereafter was raised to 1 VVM. The agitation speed during the same periods was 600 and 900 rpm, respectively. The level of dissolved oxygen tension was measured by a polarographic oxygen electrode (Ingold) and was never below 20% of the saturation value at the induction temperature. Antifoam (Adekanol LG-109) was added when necessary.

The medium used consisted of $(NH_4)_2SO_4$ (2.0 g liter^{-1}),

KH_2PO_4 (1.6 g liter^{-1}), $Na_2HPO_4 \cdot 2H_2O$ (6.6 g liter^{-1}), $(NH_4)_2$-H-citrate (0.5 g liter^{-1}), tryptophan (1.0 g liter^{-1}), proline (2.0 g liter^{-1}), glucose or glycerol (20.0 g liter^{-1}), Casamino Acids (Difco) (20.0 g liter^{-1}), 1 M $MgSO_4$ (2 ml liter^{-1}), trace component solution (14) (2 ml liter^{-1}), ampicillin (70 mg liter^{-1}), and kanamycin sulfate (20 mg liter^{-1}). At the time for temperature induction in bioreactor cultivations with controlled pH, an additional amount of either glucose or glycerol as well as Casamino Acids corresponding to the initial amounts were added. Cultures were induced at an OD_{610} of approximately 20 except in the cultivations without pH control, which were induced at an OD_{610} of 3.

Analytical methods. Cell growth was monitored by observing the OD_{610} with a spectrophotometer. Cell dry weight and acetic acid concentration were determined as described elsewhere (33). The concentration of undissociated acetic acid was calculated by using the Henderson-Hasselbalch equation, pH = pKa + log([A^-]/[HA]), assuming pKa = 4.76.

Samples for β-galactosidase (EC 3.2.1.23) assays and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were disintegrated with a high-pressure homogenizer (French press FA 073; Aminco). Disintegrated cell samples from the medium for β-galactosidase assays were immediately diluted in assay buffer to prevent inactivation of β-galactosidase by the low pH in cultivations with uncontrolled pH. The amount of SpA-βgal was measured as β-galactosidase activity (U). One unit of enzyme activity hydrolyzes 1.0 μmol of *o*-nitrophenyl-β-D-galactopyranoside min^{-1} at 25°C. The activity was determined as described elsewhere (34). The immunoglobulin G-binding activity of the protein A part in SpA-βgal has been demonstrated (24). The enzymatically active SpA-βgal as well as β-galactosidase is a tetramer. The total activity of SpA-βgal in the cultivations was expressed as units per milliliter of cultivation broth and the specific activity as units per milligram of cell dry weight. The specific production rate of SpA-βgal was expressed as units per milligram of cell dry weight per hour.

The specific activity of pure SpA-βgal (units per milligram of protein) was estimated with the following assumptions: 1 mmol of SpA-βgal has the same activity as 1 mmol of β-galactosidase, and β-galactosidase has a specific activity of 500 U mg of protein^{-1} (25°C). The molecular weight of the β-galactosidase monomer is approximately 116,000, and the molecular weight of the SpA-βgal monomer encoded by pRIT1 is approximately 148,000. This would give SpA-βgal a specific activity of 390 U mg of protein^{-1}. The SpA-βgal encoded by pRIT1b (molecular weight, 146,000) would have a specific activity of 400 U mg of protein^{-1}.

Cell samples from the cultivations for SDS-PAGE were centrifuged (2,250 × *g*, 10 min) and resuspended in 50 mM phosphate buffer (pH 7) before storage at −80°C. Disintegrated samples for SDS-PAGE were divided by centrifugation (15,000 × *g*, 10 min) into a supernatant and a cell pellet fraction. The method for preparation of samples for SDS-PAGE has been described previously (33). SDS-PAGE with a 3.5% stacking gel and an 8% separating gel was performed by the method of Laemmli (17) with the exception that the pH of the electrode buffer was 8.9. Each lane was loaded with sample corresponding to equal amounts of cells. SDS-polyacrylamide gels were scanned with a Desaga CD60 densitometer. The relative inclusion body formation was expressed as the peak area of SpA-βgal in the pellet fraction versus the sum of the corresponding peak areas of the pellet and supernatant fractions. The inclusion body concentration was calculated from the relative inclusion body formation, the total activity, and the specific activity of SpA-βgal per milligram of protein.

For N-terminal amino acid sequence analysis of SpA-βgal, inclusion body bands on SDS-polyacrylamide gels were blotted into a polyvinylidene difluoride membrane before analysis (9).

RESULTS

Effect of induction temperature. The production of SpA-βgal was studied at three different induction temperatures, 39, 42, and 44°C, and also at a combination of two temperatures, 42°C for 2 h followed by 39°C (Fig. 1). Induction and production at 44°C resulted in an increasing but low specific rate of product formation and low final activities, both total and specific. All other conditions gave a higher specific production rate of SpA-βgal which was highest during the first hours after induction and thereafter declined. Six hours after induction, the total activity of SpA-βgal was higher for cultivations with induction at 39°C (950 U ml^{-1}) compared with induction at 42°C (830 U ml^{-1}), but the specific activity was highest for the cells induced at 42°C. When cells were induced at 42°C for 2 h and then the production temperature was lowered to 39°C throughout the process, the cells grew to a higher density (14.2 g liter^{-1} after 5.5 h in the production phase) compared with production at 42°C, and both total (1,100 U ml^{-1}) and specific activities were highest in this system. A specific activity of 80 U mg^{-1} would correspond to approximately 20% of the cell dry weight, and 1,200 U ml^{-1} would correspond to a product concentration of 3 g liter^{-1}. With an induction temperature of 42°C, inclusion bodies were formed mainly during the first hours after induction, in contrast to the other induction conditions in which aggregation continued throughout the process. Induction at 39°C yielded the highest total amount of inclusion bodies at the end of the production phase, although the relative amount of inclusion body at the end of the process was comparable with that resulting from production at 44°C. In contrast, the relative amount of inclusion body formed during the first hours after induction was lowest at 39°C.

Effect of pH. We have in earlier experiments noticed a much stronger tendency toward inclusion body formation in shake flask cultures compared with cultivations in a bioreactor (33). In shake flasks, the process conditions are uncontrolled in comparison with bioreactor cultivations. Two major differences are the control of pH and aeration. To examine whether the cause of the increased inclusion body formation in shake flasks might be due to the declining pH, we performed cultivations in a bioreactor without control of pH. Cultivations were performed with two different induction temperatures, 39 and 42°C. These bioreactor cultivations were induced at the same OD_{610} as the shake flask cultures.

The total as well as the relative amount of inclusion bodies increased with the decline in pH at both induction temperatures (39 and 42°C), as shown in Fig. 2. However, the formation was much more pronounced at 42°C. The increase of the amount of inclusion bodies after the pH had dropped below 5.5 was accompanied by a decrease in the total activity of SpA-βgal.

Bioreactor cultivations in which oxygen became limiting during the production phase have also been performed but without any effect on inclusion body formation (data not shown). These cultivations were performed with controlled pH (7.0).

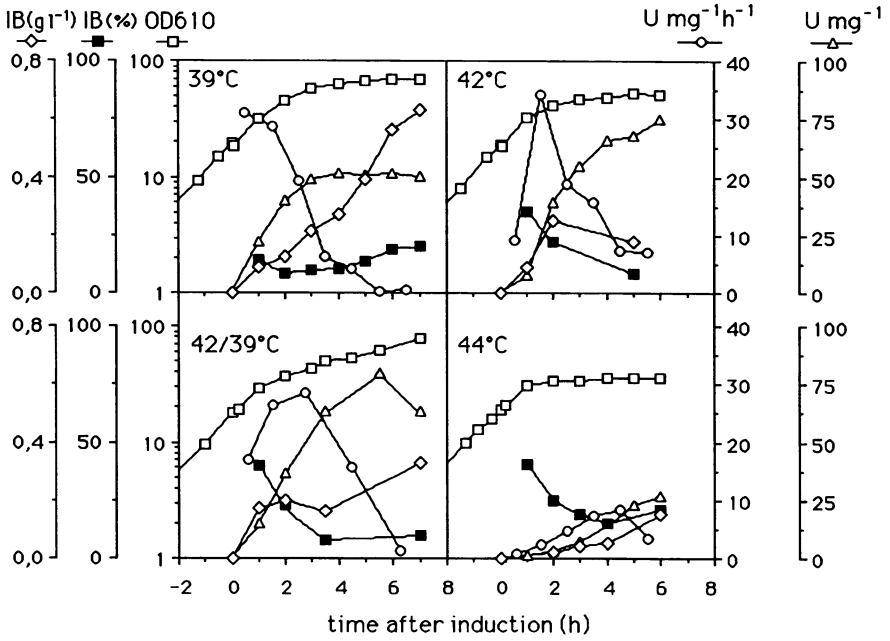

FIG. 1. Effect of induction temperature on total and relative inclusion body (IB) formation (g liter^{-1} and %, respectively), cell growth (OD_{610}), specific activity of SpA-βgal (U mg of cell dry weight^{-1}), and specific production rate of SpA-βgal (U mg of cell dry weight^{-1} h^{-1}).

Acetic acid formation. Acetic acid is known to be produced by *E. coli* as a by-product when grown on high concentrations of glucose as the carbon source. Acetic acid inhibits growth of *E. coli*. However, the inhibiting effect is not due to the total amount of acetic acid but rather to the concentration of the undissociated form of the acid (19, 29). Undissociated short-chain organic acids have the ability to penetrate the cell membrane and, when within the cell, dissociate, thereby lowering the intracellular pH. The anion might also have some unidentified inhibiting effect (5, 29). pH is also a parameter that affects the folding of a protein. Figure 3 shows acetic acid formation in cultivations with and without controlled pH (induction temperature, 42°C). Even though the total concentration of acetic acid was much higher in cultivations at pH 7.0, the concentration of undissociated acetic acid was much higher in cultivations without control of pH. We also performed cultivations at pH 7.0 (induction at 42°C) with glycerol as the carbon source since growth on glycerol is known to produce less acetic acid than growth on glucose (1). At 30°C, low amounts of acetic acid were produced with growth on glycerol, but after temperature induction, substantial amounts of acetic acid were produced from glycerol, although at a lower rate compared with growth on glucose. The specific production rate of acetic acid from glycerol was approximately 70% of the rate from glucose. The relative formation of inclusion bodies was also lowered to the same extent (data not shown).

Effect of changes in the linker region of the protein. During the work on proteolytic degradation of SpA-βgal encoded by pRIT1 (11), a new SpA-βgal was constructed with an altered linker region (9). This SpA-βgal was encoded by pRIT1b. A stretch of 41 amino acids (-GAWLLPVSLVKRKTTLAP NTQTASPRALADSLMQLARQVSR-) of 48 in the linker region was replaced by 8 amino acids (-VDLQPSLA-). Thirty-eight of the replaced amino acids in the linker region (underlined) originated from the C terminal of the *lac* repressor protein. The new SpA-βgal also contained 17 more amino acids of the N terminal of β-galactosidase. We found that the pRIT1b-encoded SpA-βgal did not form any inclusion bodies under the different conditions that produced inclusion bodies with pRIT1 although specific activities up to 125 U mg of cell dry weight^{-1} could be achieved. This corresponded to approximately 30% of the cell dry weight.

N-terminal sequence analysis of pRIT1-encoded SpA-βgal from soluble protein and from inclusion bodies showed that the terminal sequences corresponded. The N-terminal sequence was also in accordance with the predicted one (data not shown).

DISCUSSION

The formation of inclusion bodies may proceed through nonpermissive pathways from folding intermediates during the folding process as suggested by Mitraki and King (21), and temperature is one parameter that affects the conformation and stability of proteins (6, 27). Increased temperature has been found to stimulate aggregation in several cases (3, 4, 31). The effect of the induction temperature on the formation of inclusion bodies of SpA-βgal also indicates that the formation might be caused by hydrophobic interactions between protein chains. A higher temperature increases hydrophobic interaction and might also expose hydrophobic stretches of amino acids that are normally not exposed. The lack of inclusion body formation after changes in the amino acid sequence in the linker region between protein A and β-galactosidase also indicates that the folding of SpA-βgal may be important for the formation of a soluble protein. An alternative explanation would be specific intermolecular interactions of the linker region of SpA-βgal from pRIT1. Thirty-eight of the amino acids in the linker region originate from the C terminal of the *lac* repressor protein, LacI. The C terminal of LacI is not involved in DNA binding but might be

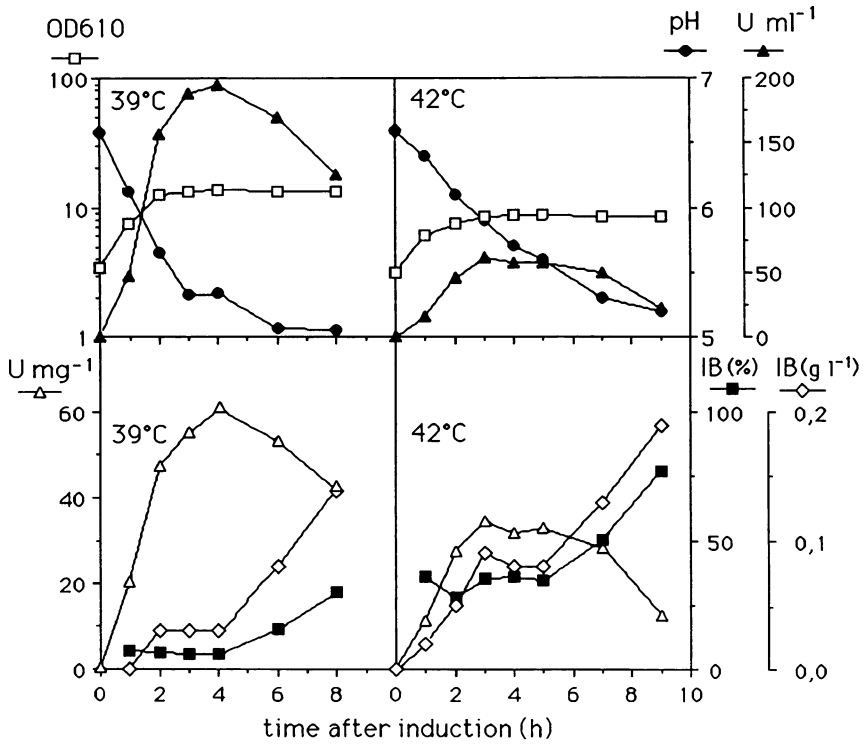

FIG. 2. Effect of decreasing pH in cultivations with induction at 39 and 42°C. The figure shows total and relative inclusion body (IB) formation (g liter^{-1} and %, respectively), cell growth (OD_{610}), total and specific SpA-βgal activities (U ml^{-1} and U mg of cell dry weight^{-1}, respectively), and pH during the production period.

involved in binding of inducer or in the formation of its tetrameric structure (2). The linker region of pRIT1-encoded SpA-βgal is exposed since it is subjected to proteolytic cleavage in the linker by the outer membrane-bound protease OmpT during purification (9). The linker also contains regions rich in hydrophobic amino acids. Lee and coworkers (18) have shown that when a hydrophobic sequence is introduced between β-galactosidase and a region of the hepatitis B virus surface antigen, inclusion body formation increases with the incubation temperature. The fusion protein without the hydrophobic sequence remained soluble independent of the incubation temperature.

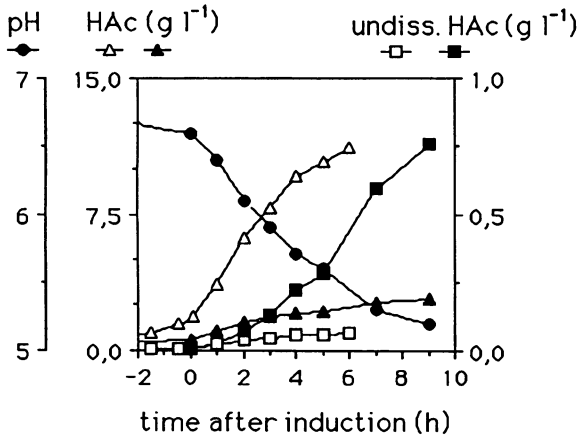

FIG. 3. Comparison of the concentrations of total and undissociated acetic acid (HAc) in a cultivation at pH 7.0 (open symbols) and in a cultivation without control of pH (filled symbols). Induction temperature was 42°C.

The reason why the inclusion body formation mainly takes place during the first hours after induction at 42°C but continues throughout the process at 39°C might be explained by functional heat shock proteins called chaperonins (15, 26). They are thought to interact with misfolded proteins and thereby influence folding or proteolysis. During the first hour after induction, the level of chaperonins relative to the level of misfolded SpA-βgal might be too low to interact with all of the misfolded protein since the production rate of SpA-βgal was highest during the first hours after induction. The level of the heat shock proteins after a thermal shock is dependent on the degree of the heat shock. The steady-state level of at least some heat shock proteins varies about twofold between 37 and 42°C, but transiently, the levels are higher after a heat shock (12, 22). Thus, a lower temperature rise gives a lower response. The continuous production of inclusion bodies at 39°C compared with the transient production at 42°C might be due to a difference in the level of heat shock proteins or the level of chaperonins. Overexpression of chaperonins together with proteins that misfold in bacteria has been suggested as a method to prevent improper folding (15). DnaK, one of the heat shock proteins, has been shown to be able not only to protect RNA polymerase against heat inactivation but also to reactivate aggregated RNA polymerase (32). DnaK has also been shown to bind to the C terminus of a truncated protein A, which corresponds to the protein A part of SpA-βgal (10). In addition, the two heat shock proteins GroEL and GroES were found to promote the assembly of a heterologous protein expressed in *E. coli*

(8). GroEL and GroES could also reconstitute the same protein in vitro after it had been denaturated with urea, guanidine-HCl, or acid (7).

Changes in intracellular pH would also influence the folding process and conformation of proteins. The results from the cultivations with declining pH show that as the pH declined, the amount of inclusion bodies increased. As the pH is declining, the cells will have increasing difficulties in maintaining the intracellular pH constant. A change in external pH from 7.0 to 5.0 has been reported to decrease the intracellular pH of 7.7 in *E. coli* by 1 unit (13). The concentration of undissociated acetic acid in the cultivations without control of pH was much higher than that in the cultivations at pH 7 even though the reverse was true for the total concentration of acetic acid. A total of 30 mg of undissociated acetic acid liter^{-1}, corresponding to approximately 5 g of total acetic acid liter^{-1} at pH 7.0, has been reported to affect growth of *E. coli* in an inhibition model suggested by Luli and Strohl (19). The model was also in accordance with experimental data.

The facts that the fraction of inclusion bodies increased when pH declined below 5.5 and the total amount of soluble SpA-βgal declined at the same time indicate that the inclusion bodies could be formed from already soluble and active protein and not only at the initial folding after translation. The decline of soluble and enzymatically active SpA-βgal could be monitored both by a decline in SpA-βgal activity and by the weakening of the band corresponding to soluble SpA-βgal on SDS-polyacrylamide gels. If the decline in the amount of soluble SpA-βgal was not caused by inclusion body formation, the only explanation for the decline is a proteolytic degradation of SpA-βgal. However, no bands corresponding to degradation products were found on SDS-polyacrylamide gels to support this explanation.

The specific production rate of acetic acid was highest just after induction and declined thereafter with time. This high initial production rate after induction might also influence the formation of inclusion bodies, since using glycerol as the carbon source reduced the specific production rate of acetic acid as well as the formation of inclusion bodies.

The highest total and specific activities of pRIT1-encoded SpA-βgal after 6 h of induction were achieved with induction at 42 and then 39°C, reaching about 1,100 U ml^{-1} and 80 U mg^{-1}, respectively. Somewhat lower total activity, 950 U ml^{-1}, was obtained with induction at 39°C, but the specific activity was only 50 U mg^{-1}. Induction at 44°C gave a low production of both SpA-βgal and cell mass. The low activities found at 44°C cannot be explained solely by an increased inclusion body formation. The low production rate of SpA-βgal could be caused by an increased mRNA instability or by energy limitation caused by the thermal stress. Increasing the induction temperature also yielded a lower final cell mass. Choosing appropriate temperature induction conditions is important not only for both total and specific yields of recombinant proteins but also for plasmid stability and product homogeneity (25). In our experiments, plasmid segregation and product degradation did not pose any problem.

This study shows that by choosing appropriate cultivation conditions we could either produce or avoid inclusion bodies. Production temperature, extracellular pH, and the carbon source influenced inclusion body formation. The induction temperature was also important for optimizing the product yield. This emphasizes the importance of careful examination of the cultivation conditions for the production of recombinant proteins in order to control inclusion body formation. The effect of small changes in the amino acid sequence on inclusion body formation also emphasizes the importance of the protein structure. The fact that the inclusion body formation increases initially with increased temperature further supports this. The declining tendency of aggregation with time at 42°C but not at 39°C indicates that heat shock proteins may be involved in protection against inclusion body formation from incorrectly folded proteins or intermediates. The formation from existing soluble protein observed at declining pH indicates also that inclusion bodies may not always be formed from folding intermediates.

ACKNOWLEDGMENTS

We thank Mathias Uhlén with coworkers and Halldis Hellebust for providing the different plasmids. We also thank Eva Hedblom and Stefan George for technical assistance during parts of this work.

This investigation was supported by the Swedish National Board for Technical Development.

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