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Characterization of up-regulated proteases in an industrial recombinant *Escherichia coli* fermentation

GL Jordan and SW Harcum

Department of Chemical Engineering, New Mexico State University, Las Cruces, NM 88003, USA

Proteolytic degradation of recombinant proteins is an industry-wide challenge in host organisms such as *Escherichia coli*. These proteases have been linked to stresses, such as the stringent and heat-shock responses. This study reports the dramatic up-regulation of protease activity in an industrial recombinant *E. coli* fermentation upon induction. The objective of this project was to detect and characterize up-regulated proteases due to recombinant AXOKINE® overexpression upon IPTG induction. AXOKINE® is a 22-kDa protein currently in clinical trials as a therapeutic for obesity associated with diabetes. AXOKINE® was expressed in both the soluble and inclusion body fractions in *E. coli*. Sodium dodecyl sulfate gelatin–polyacrylamide gel electrophoresis (SDS-GPAGE) was used to analyze the up-regulated protease activity. Western blot analysis showed degraded AXOKINE® in both the soluble and insoluble fractions. Protease inhibitors were used to characterize the proteases. The proteases were ethylenediaminetetraacetic acid (EDTA) sensitive. The protease activity increased in the presence of phenyl-methyl sulfonyl-fluoride (PMSF), a serine protease inhibitor. The incubation buffer composition was varied with respect to Mg^{2+} and ATP, and the protease activity was ATP independent and Mg^{2+} dependent. A two-dimensional electrophoresis technique was used to estimate the pI of the proteases to be between 2.9 and 4.0.
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Introduction

In recombinant *Escherichia coli* that overproduce a recombinant protein, elevated protease activity has been observed that resulted in product loss [5,31]. These proteases included OmpT, La, and 34/39-kDa proteases [1,4,6,12,20,21,29]. Recently, Gill *et al* [4] have identified the 34/39-kDa protease to be OmpT, based on the affinity of the 34/39-kDa protease to an OmpT antibody. The La and OmpT proteases are also induced by a variety of other stresses [3,7,10,26,31]. Protease La, a 360-kDa tetramer with identical 90-kDa subunits, was the first protease to be observed to degrade recombinant proteins. La was initially identified as a heat-shock protein that degraded abnormal proteins at the expense of ATP [6–8]. Thus, protease La activity was attributed to the abnormal appearance of the recombinant protein [6]. OmpT protease activity is up-regulated by the heat-shock and stringent responses [12,14]. OmpT is an outer membrane-associated protease with a molecular weight of 33.5-kDa. OmpT has been characterized as a serine protease that is ethylenediaminetetraacetic acid (EDTA) insensitive and phenyl-methyl sulfonyl-fluoride (PMSF) sensitive [17,18,28]. OmpT protease activity has been observed in inclusion bodies [18]. Also, OmpT has been reported to have proteolytic activity under denaturing conditions [32]. OmpT protease activity has also been correlated with recombinant protein loss of a soluble recombinant protein, chloramphenicol acetyltransferase (CAT) [12–16]. Additionally, micronutrient amino acid feeding was shown to reduce the induced OmpT protease activity and improve recombinant CAT productivity where CAT was located only in the soluble fraction [22–24].

In this study, the objective was to characterize the protease activity up-regulated upon IPTG induction, in this case by the overproduction of AXOKINE®. AXOKINE® is a 22-kDa recombinant protein currently in clinical trials as a therapeutic for obesity associated with diabetes, which has demonstrated efficacy in a Phase 2 clinical trial. Western blot analysis was used to confirm AXOKINE® degradation during the fermentation for both the soluble and insoluble fractions. Sodium dodecyl sulfate gelatin–polyacrylamide gel electrophoresis (SDS-GPAGE) gels were used to characterize the protease activity in the presence of protease inhibitors. The incubation buffer cofactor (Mg^{2+} and ATP) requirements were also investigated. Additionally, a two-dimensional (2D) electrophoresis-based gel analysis was used to detect protease activity, termed a 3D gel. The 3D gel was used to determine the protease pI via protease activity identified by clear zones on the gel due to gelatin degradation.

Materials and methods

E. coli RFJ141 [pRG421] cells that produce AXOKINE® were supplied by Regeneron Pharmaceuticals, of Tarrytown, NY. *E. coli* RFJ141 is a W3110 $lacI^q$ derived strain. The pRG421 plasmid is a high-copy *lacUV5* expression vector containing the gene for kanamycin resistance. Cells were harvested from a 150-l fed-batch culture grown in defined media. AXOKINE® expression was induced by the addition of IPTG to the culture. The *E. coli* cells (preinduction, 2-, 4-, 6-, and 8-h postinduction) were shipped on ice, overnight from Regeneron to New Mexico State University (NMSU), Las Cruces, NM. The cells were not shipped on dry ice to avoid AXOKINE® precipitation. Studies conducted at Regeneron

Correspondence: Dr Sarah W. Harcum, Department of Chemical Engineering, New Mexico State University, Las Cruces, NM 88003, USA
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demonstrated that for the proteases of interest, activity could be restored after overnight storage on ice. Upon arrival at NMSU, the cells were centrifuged at 3000 rpm for 45 min at 4°C to separate the medium from the cells. Cells were resuspended in a Tris–HCl dithiothreitol (TDTT) buffer (equal volumes) and stored at −55°C [27]. Cell suspensions were thawed overnight on ice and sonicated on ice for 30 s to lyse cells. The sonicated samples were centrifuged at 3000 rpm for 45 min at 4°C to separate the soluble and insoluble fractions. Soluble fractions were stored at −55°C. Insoluble fractions were resuspended in 40 ml 6 M urea per gram insoluble material and stored at −55°C. The total protein concentration of the samples was determined by the total protein assay (Bio-Rad) at 595 nm [27].

Western blots on nitrocellulose membranes were probed with a rabbit anti-human CNTF (ciliary neurotropic factor) monoclonal antibody to detect AXOKINE®. The amount of sample loaded was 20 μg protein per lane. A goat anti-rabbit polyclonal antibody linked to alkaline phosphatase (AP) was used to probe the primary antibody and the membrane was then incubated with streptavidin–AP to reveal AXOKINE®.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis gels (SDS-PAGE) and SDS-GPAGE gels were prepared as described by Harcum and Bentley [13], except multicast linear gradient gels (10% to 20%) were used (Bio-Rad's Model 385 Gradient Former and Mini-PROTEAN® II Multi-Casting Chamber with 1-mm spacers were used). SDS-PAGE gels do not contain gelatin and cannot detect protease activity. Protease activity was detected on SDS-GPAGE gels as clear zones or bands, which appeared as white bands on a dark background. The SDS-PAGE and SDS-GPAGE gels were stored at 4°C for up to 2 weeks. Durkee red food coloring (5 μl per gel) was used to differentiate the stacking gel from the electrophoresis buffer. SDS-PAGE and SDS-GPAGE gels were run at 200 V (constant voltage) for approximately 1 h using a PowerPac 1000.

The Mini-PROTEAN® II 2D Tube Cell (Bio-Rad) apparatus and protocol were used for 2D and 3D gels, except that β-mercaptoethanol was omitted to reduce protein denaturation. Isoelectric focusing (IEF) tube gels (30% T and 5.4% C) were cast and stored at 4°C for up to 2 weeks. The IEF gels were typically loaded with 20 μg protein and run at 750 V (constant voltage) for 4 h using a PowerPac 1000 (Bio-Rad) power supply. For 2D gels, the IEF tube gels were placed on SDS-PAGE gels. For 3D gels, the IEF tube gels were placed on SDS-GPAGE gels [16]. The first dimension (IEF gels) separated proteins by isoelectric point, the second dimension (SDS-PAGE or SDS-GPAGE gels) separated proteins by molecular weight, and the third dimension (3D gels) detected protease activity. The 2D and 3D gels were run at 200 V for 1 h. The 2D gels were immediately silver stained using the Silver Staining Plus Kit (Bio-Rad). The SDS-GPAGE and 3D gels were washed in Triton X-100 for 1 h to remove SDS, then washed in MilliQ water for 5 min to remove Triton X-100 from the gels [13]. SDS-GPAGE and 3D gels were incubated for 24 h at 37°C in an incubation buffer. The incubation buffers (pH 7.0) used were: Buffer 1 (100 mM glycine, 2 mM ATP, and 2 mM $MgCl_2$); Buffer 2 (100 mM glycine and 2 mM $MgCl_2$); Buffer 3 (100 mM glycine and 2 mM ATP); and Buffer 4 (100 mM glycine). Buffer 1 was used for all incubations, unless otherwise specified. After incubation, the SDS-GPAGE and 3D gels were removed from the buffer and stained with Amido Black for 1 h and destained for approximately 4 h to reveal clear zones due to protease activity [13]. All stained gels were scanned with the Densitometer model GS-700 (Bio-Rad) and analyzed by Quantity One® (Bio-Rad) software.

Protease inhibitors were added to the incubation buffer (Buffer 1) to characterize the proteases on SDS-GPAGE gels. Protease inhibitors were from Sigma Chemicals. EDTA (500 mM in MilliQ water, pH >8) and PMSF (100 mM in dimethyl sulfoxide [DMSO]) stock solutions were stored at room temperature. Pepstatin A (1 mM in DMSO) stock solution was stored at 4°C. Chymostatin (10 mM in DMSO) and antipain (10 mM in MilliQ water) stock solutions were stored at −20°C. Benzamindine was prepared fresh each time. The incubation buffer protease inhibitor concentrations were 0.165 mM antipain, 100 mM benzamindine, 0.165 mM chymostatin, 50 mM EDTA, 0.015 mM pepstatin A, and 1.0 mM PMSF unless otherwise specified. The inhibitor concentrations used are typical concentrations used for protease characterization [16].

Results and discussion

Protease activity induction

Western blots of soluble and insoluble fractions showed that AXOKINE® was degraded (Figure 1). Figure 1 shows Western blots of the soluble and insoluble fractions for two separate AXOKINE® fermentations at 2-, 4-, 6-, and 8-h postinduction. A soluble preinduction fraction sample was loaded in Lane 10 on both Western blots. Full-length AXOKINE® is the upper band in each panel (22-kDa). The 18- and 14-kDa band proteins are degraded forms of AXOKINE®. The amount of degraded AXOKINE® increased with time postinduction, as indicated by the increased intensity of the lower molecular weight bands (18- and 14-kDa) for the insoluble fractions. The 14-kDa degraded AXOKINE® band appeared earlier with a higher intensity in the soluble fraction (2-h postinduction) compared to the insoluble fraction. The amount of the 14-kDa degraded AXOKINE® remained relatively constant in the soluble fraction for the 2- to 8-h postinduction samples, whereas the intensity of the 14-kDa degraded AXOKINE® band in the insoluble fraction increased with time postinduction for the 2- to 6-h postinduction samples. The 14-kDa degraded AXOKINE® band in the insoluble fraction for the 8-h postinduction samples are similar to the 6-h postinduction samples. Additionally, an 18-kDa degraded AXOKINE® band appeared in the insoluble fractions for the 4-, 6-, and 8-h

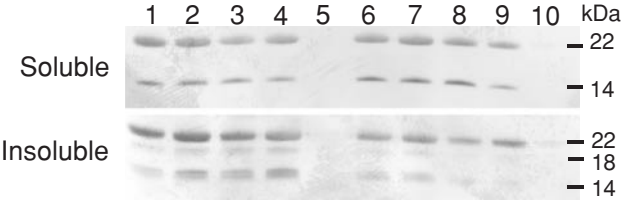

Figure 1 Western blot of AXOKINE® from the soluble and insoluble fractions of recombinant *E. coli.* Two separate fermentations are shown. The first is shown in lanes 1, 3, 6, 8, and 10 and the second in lanes 2, 4, 7, and 9. Lane 5 is a molecular weight marker. All lanes were loaded with 20 μg protein. Lanes 1 and 2 were 8-h postinduction, lanes 3 and 4 were 6-h postinduction, lanes 6 and 7 were 4-h postinduction, lanes 8 and 9 were 2-h postinduction, and lane 10 was preinduction. Soluble fraction samples are shown in the top panel and insoluble fraction samples in the bottom panel. Full-length AXOKINE® is 22-kDa. Degraded forms of AXOKINE® are 14- and 18-kDa.

postinduction samples. The 18-kDa degraded AXOKINE® band was not observed in any of the soluble fractions.

Other researchers demonstrated that IPTG does not induce protease activity [12,14]. Therefore, the degradation of AXOKINE® was due to proteases that were up-regulated by the overexpression of AXOKINE®. Interestingly, the amount of AXOKINE® degradation was higher for the insoluble fraction. This higher degradation rate could be attributed to either, AXOKINE® was degraded in the insoluble fraction, or degraded AXOKINE® was more likely to form insoluble material. The appearance of the 18-kDa degraded AXOKINE® band could be attributed to different protease activity in the insoluble and soluble fractions. This study's objective was limited to characterization of the up-regulated protease activity, and not to determine the origin of the higher levels of degraded AXOKINE® in the insoluble fraction. However, the Western blots clearly indicated that AXOKINE® was degraded *in vivo*, potentially by the observed up-regulated protease activity.

In addition to the AXOKINE® Western blots, SDS-GPAGE gels were used to analyze the protease activity up-regulation. SDS-GPAGE gels indicated that protease activity was up-regulated by AXOKINE® overexpression. The protease activity increased with time postinduction for both the soluble and insoluble fractions (data not shown). The postinduction samples had significantly more protease activity compared to uninduced samples on a protein basis. The insoluble fractions had more protease activity compared to the soluble fractions on a protein basis. Protease activity was observed as a series of clear zones or bands with molecular weights between approximately 35- and 42-kDa (Figure 2A, control). The most prominent protease was observed as a clear band with a molecular weight of approximately 35-kDa. From the protease activity data obtained on the SDS-GPAGE gels, it cannot be determined if the series of clear bands observed were due to a single protease or to multiple proteases. However, multiple clear bands have been observed for purified proteases on SDS-GPAGE gels, specifically, trypsin, elastase, and collagenase [11]. Also, the observed protease activity behavior for all of the bands was similar in the presence of protease inhibitors and cofactors (Figure 2A). The clarity and consistency of the 35-kDa protease activity band allowed for reproducible quantification. Therefore, the 35-kDa protease band was selected to quantify up-regulated protease activity, as a representative up-regulated protease for this study. Based on the Western blot and time series SDS-GPAGE gels, the 6-h postinduction sample was selected as a representative sample to be used to characterize the protease activity, and specifically, the 35-kDa protease.

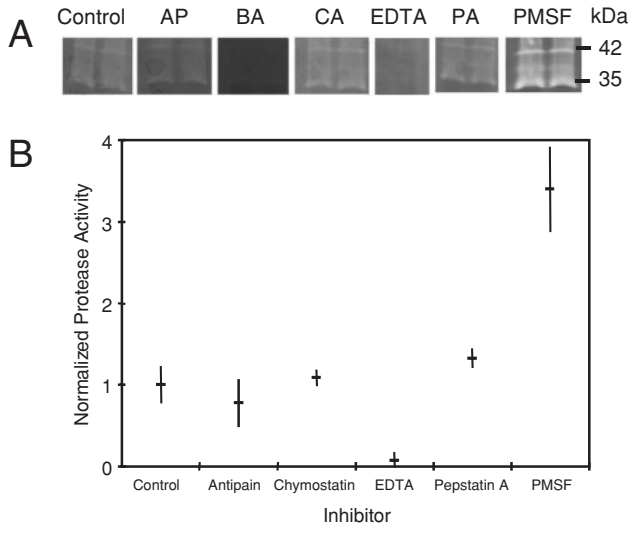

Figure 2 SDS-GPAGE of 6-h postinduction AXOKINE® insoluble sample from recombinant *E. coli* incubated with different protease inhibitors (Panel A). Samples were separated on a 10–20% linear gradient SDS-GPAGE. Protease inhibitors were added to the incubation buffer to determine protease inhibition. Control: Buffer 1; AP: 0.165 mM antipain, BA: 100 mM benzamidine; CA: 0.165 mM chymostatin; EDTA: 50 mM EDTA; PA: 0.015 mM pepstatin A; and PMSF: 1 mM PMSF. All lanes were loaded with 15 μg protein, in duplicate. Gels were stained with Amido Black. The up-regulated proteases have molecular weights between 35- and 42-kDa, where the major protease has a molecular weight of 35-kDa. Panel B: the normalized 35-kDa protease activity for the set of three SDS-GPAGE gels. The protease activity was normalized to the control with 95% confidence intervals indicated.

The observation of significant protease activity associated with the insoluble fractions supports the hypothesis that AXOKINE® was potentially degraded in the insoluble fractions. Currently, some industrial recombinant proteins are purposely produced in inclusion bodies, or the insoluble fraction, as a means to reduce purification steps. This study suggested that inclusion body recombinant proteins are not protease protected, as previously considered [31], and may not be the best production methods for some recombinant proteins.

Protease characterization with protease inhibitors

In order to characterize the proteases found to be up-regulated by AXOKINE® overexpression, six protease inhibitors were used. The concentration of the inhibitors selected for these studies were standard concentrations [4,16,17,20,28–30,32]. Protease inhibitors used were antipain, benzamidine, chymostatin, EDTA, pepstatin A, and PMSF [16]. The protease inhibitors were added to the incubation buffer for the SDS-GPAGE gels. The SDS-GPAGE gels were loaded with 6-h postinduction insoluble sample at 15 μg protein per lane, in duplicate. Three sets of gels were used for the analysis. Figure 2A contains photographs for a set of gels that show the effects of the protease inhibitors on protease activity as detected by SDS-GPAGE. Protease activity was detected as clear zones or bands on dark backgrounds. The proteases observed were between 35- and 42-kDa, where the 35-kDa protease was the prominent protease band. Previous researchers demonstrated that the clear zones are proportional to the amount of protease present in the gel [13]. From Figure 2A, it was observed qualitatively that EDTA inhibited the proteases compared to the control. Antipain (AP), chymostatin (CA), and pepstatin A (PA) had the same amount of protease activity compared to the control. The PMSF lanes appeared to have greater protease activity compared to the control. Note in Figure 2A, all the protease activity clear zones decreased or increased together in the presence of the various protease inhibitors, which suggested that the series of protease bands could be the result of a single protease. Multiple bands have been observed for purified commercial proteases (trypsin, elastase, and collagenase) on SDS-GPAGE gels [11]. Figure 2B is a quantitative summary of the 35-kDa protease activity data obtained from the three sets of gels for each of the protease inhibitors shown in Figure 2A. Figure 2B shows the normalized 35-kDa protease activity for the 6-h postinduction insoluble sample incubated with protease inhibitors, normalized to the control lanes with the 95% confidence intervals (error bars with two standard deviations)

shown. The clear zone at 35-kDa was the only band quantified. A single-factor ANOVA test determined that the protease inhibitors were a significant parameter affecting the 35-kDa protease activity ($p<0.0001$). A paired two-sample t-test was used to compare the protease inhibitors to each other and the control. The paired t-tests indicated that PMSF and EDTA were significantly different from the control and the other three protease inhibitors ($p<0.02$). The antipain, chymostatin, and pepstatin A protease inhibitors did not result in significantly different protease activity compared to the control ($p>0.05$). Benzamindine altered the staining and destaining of the gel, such that no conclusion about its inhibitory effect could be made. EDTA significantly inhibited the 35-kDa protease activity ($p<0.002$). PMSF, a known serine protease inhibitor, significantly enhanced protease activity ($p<0.006$). The 35-kDa protease appeared to degrade the gelatin to a greater extent when PMSF was in the incubation buffer.

The unexpected result of PMSF enhancing protease activity was further investigated by using various levels of PMSF in the incubation buffer. The incubation buffer PMSF concentrations were varied from 0 to 5.0 mM. Protease activity was normalized to the control (Buffer 1 without PMSF). Figure 3 shows the increase in normalized 35-kDa protease activity with PMSF concentrations (0 to 5.0 mM) in the incubation buffer, with the 95% confidence intervals shown. A single-factor ANOVA test determined that the PMSF concentration was a significant parameter affecting the 35-kDa protease activity ($p<0.0001$). A paired t-test was used to compare the different PMSF concentrations to each other. The paired t-test indicated that the 0.05 mM and 0.1 mM PMSF samples were not significantly different from the control or each other ($p>0.05$). Additionally, the paired t-test indicated that the 1.0 mM and 5.0 mM PMSF samples were not significantly different from each other ($p>0.05$), but that the 1.0 mM and 5.0 mM PMSF samples were significantly different from the control, 0.05 mM, and 0.1 mM PMSF samples ($p<0.001$). The PMSF concentration significantly enhanced the 35-kDa protease activity for concentrations 1.0 mM PMSF and higher.

The only protease inhibitors that significantly affected the up-regulated protease activity were EDTA and PMSF. EDTA inhibited the 35-kDa protease, whereas PMSF resulted in greater protease activity on the SDS-GPAGE gels. EDTA inhibition of the 35-kDa protease indicated that the up-regulated proteases require a cation for optimal activity, and therefore are likely to be methalloproteases [25]. The behavior of the up-regulated protease activity in the presence of the protease inhibitors was significantly different from the reported behavior of OmpT [4,12,14,15]. Specifically, OmpT has been reported to not be inhibited by EDTA [4,19,29].

Antipain, chymostatin, pepstatin A, and PMSF typically inhibit serine proteases. The up-regulated 35-kDa protease, in this study, was not inhibited by these four serine protease inhibitors. Therefore, the up-regulated proteases are unlikely to be typical serine proteases [25]. OmpT has been reported not to be inhibited by these four serine protease inhibitors [4], yet OmpT is considered a serine protease. Thus, the up-regulated proteases might be serine proteases, but would have OmpT-like serine protease characteristics. Pepstatin A has been reported to inhibit aspartic proteases; therefore, the up-regulated proteases are unlikely to be aspartic proteases, since the 35-kDa protease activity was unchanged by pepstain A.

The 35-kDa protease activity increased in the presence of PMSF. The unexpected result of PMSF enhancing the 35-kDa protease activity was even more interesting, when it was determined that the 35-kDa protease activity enhancement was proportional to the PMSF concentration. Double reciprocal plots, or Lineweaver–Burk plots, of the normalized protease activity and the PMSF concentration indicated that PMSF acted as a substrate or cofactor for the protease activity reaction (graph not shown). This is the first known report of an *E. coli* protease having enhanced protease activity in the presence of increasing amounts of a protease inhibitor. The mechanism of protease activity enhancement by PMSF for the 35-kDa protease could prove interesting, however was not part of this study's objectives.

OmpT has been reported to be inhibited by 5.0 mM PMSF [25,29]. Other authors have reported that OmpT was not inhibited by lower PMSF concentrations [28,32]. Additionally, no reports have indicated increased protease activity for OmpT in the presence of PMSF. The EDTA protease inhibition and PMSF enhancement data for the major up-regulated proteases suggest that these proteases are not OmpT. The protease activity data presented by this study does not exclude that OmpT might have been detected by the SDS-GPAGE analysis techniques, just that OmpT was not the major or only up-regulated protease detected.

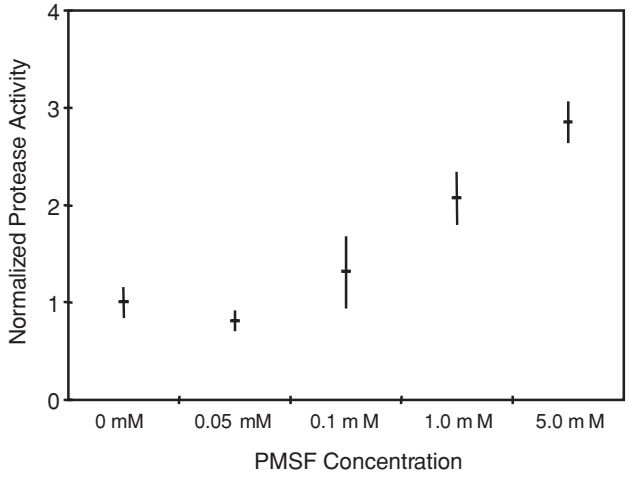

Figure 3 Normalized 35-kDa protease activity for 6-h postinduction AXOKINE® insoluble sample incubated with different PMSF concentrations with 95% confidence intervals indicated.

Incubation buffer cofactors

In order to evaluate the dependence of the up-regulated proteases on various cofactors, SDS-GPAGE gels were contacted with the four different incubation buffers. The SDS-GPAGE gels were loaded with 15 μg protein per lane of 6-h postinduction insoluble sample. Figure 4 compared the 35-kDa protease activity in the presence of different incubation buffers containing Mg^{2+} and ATP. The normalized 35-kDa protease activities for the four incubation buffers are shown in Figure 4, with the 95% confidence intervals indicated. A single-factor ANOVA test determined that the incubation buffer composition was a significant parameter affecting the 35-kDa protease activity ($p<0.0005$). A paired two-sample t-test was then used to compare the buffers to each other. The paired t-test indicated that Buffers 3 and 4 (no Mg^{2+}) were not significantly different ($p>0.05$) from each other. Also, the paired t-test indicated that Buffers 1 and 2 (both containing Mg^{2+}) were not significantly different ($p>0.05$) from each other.

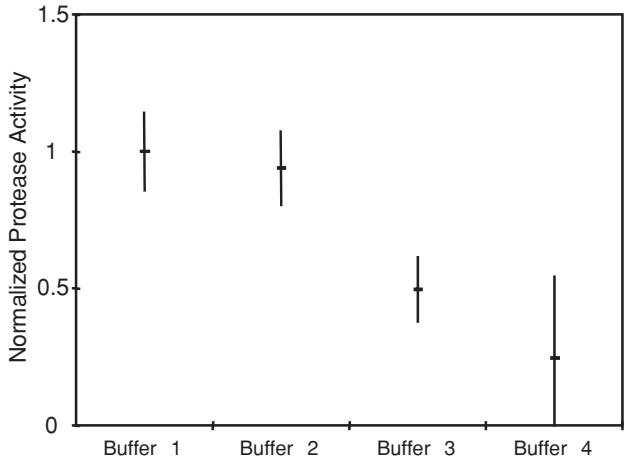

Figure 4 Normalized 35-kDa protease activity for 6-h postinduction insoluble AXOKINE® sample incubated in four different incubation buffers. The protease activity was normalized to the control (no PMSF) with 95% confidence intervals indicated. Buffers 1 and 2 contained Mg^{2+}, while Buffers 3 and 4 did not contain Mg^{2+}.

However, the paired *t*-test indicated Buffers 3 and 4 were statistically different from Buffers 1 and 2 ($p<0.02$). Buffers 1 and 2 resulted in higher observed 35-kDa protease activity compared to Buffers 3 and 4. The major component difference between the four buffers was the ATP and $MgCl_2$ content. Buffer 1 contained ATP and Mg^{2+}, Buffer 2 contained Mg^{2+}, Buffer 3 contained ATP, and Buffer 4 contained only the buffering agent, glycine. Thus, the 35-kDa protease required $MgCl_2$ for optimal activity, but did not require ATP for activity.

Many proteases in *E. coli* require ATP and/or Mg^{2+} for activity. Thus, it was important to determine the ATP and Mg^{2+} requirements for the 35-kDa protease. Based on the SDS-GPAGE gels, Mg^{2+} was required for optimal protease activity and ATP was not required for optimal protease activity. EDTA inhibition of the 35-kDa protease and Mg^{2+} requirement data, together, indicated that the up-regulated 35-kDa protease may be a metalloprotease.

The most highly studied protease associated with abnormal protein degradation is La. La is ATP and $MgCl_2$ dependent. It does not exhibit protease activity on the SDS-GPAGE gels [11]. Thus, the up-regulated protease activity observed in this study, has significantly different protease activity behavior than La [6]. The 35-kDa protease does not match any of the known *E. coli* proteases identified to date [21,26]. Thus, it appears that there is at least one more unidentified protease in *E. coli* that is up-regulated by recombinant protein overexpression.

Since proteolytic degradation of recombinant proteins is an industry-wide challenge, EDTA could be used to control protease activity of the 35-kDa protease during downstream processing, relatively inexpensively. In fact, EDTA is a very common component of bioprocessing buffers. Thus, at the downstream processing level, the 35-kDa protease is not likely to be responsible for recombinant protein degradation. However, the 35-kDa protease could be responsible for recombinant protein degradation *in vivo*.

Understanding protease activity that is up-regulated by recombinant protein production will allow for better control of fermentations and downstream processing. Three approaches could be used to control protease activity: (1) prevention of the stress in cells that causes protease up-regulation, (2) usage of protease inhibitors in downstream purification buffers, and (3) deletion of the protease genes from the host organism. Thus far, only protease La and OmpT have been specifically identified as proteases that degrade abnormal or recombinant proteins in *E. coli*. La and/or OmpT deficient *E. coli* strains have been used to produce recombinant proteins [6,17,20,30]. However, these protease deficient strains still had detectable protease activity and quantifiable recombinant protein degradation [2,20,30,31]. Additionally, many of the protease deficient strains do not grow robustly enough for commercial recombinant protein production [31]. Another approach to eliminate or reduce protease activity in *E. coli* would be to determine the underlying mechanism that induces protease activity and use bioprocess control to control protease activity. For example, OmpT (known as the 34/39-kDa protease) is up-regulated by recombinant protein overexpression [12]. The OmpT protease is induced by a stringent-like response, that in part, could be controlled by micronutrient amino acid feeding [4,12,14,22–24].

Determination of protease pI

In order to determine the pI of the 35-kDa protease, a 3D gel was used to locate the protease by protease activity in a 2D gel format. A 6-h postinduction insoluble fraction sample was separated on paired 2D and 3D gels. Figure 5 shows the 2D and 3D gels for the

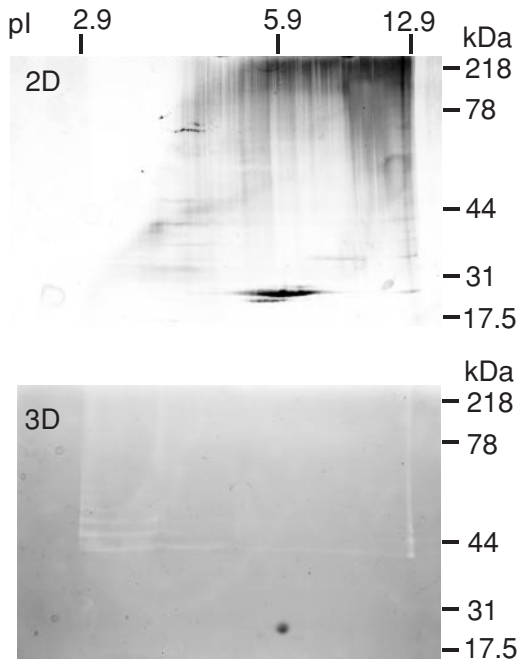

Figure 5 2D and 3D gels (top and bottom panels, respectively) for 6-h postinduction AXOKINE® insoluble sample from recombinant *E. coli*. The proteins were separated on a 10–20% linear gradient SDS-PAGE or SDS-GPAGE, for the 2D and 3D gels, respectively. The gels were loaded with 20 μg protein. The 2D gel was silver stained and the 3D gel was stained with Amido Black. AXOKINE® was visible as a large spot on the 2D gel with a molecular weight of 22-kDa at pI 5.92. The 35-kDa protease was not visible on the 2D gel. AXOKINE® was visible as a spot on the 3D gel with a molecular weight of 22-kDa at pI 5.92. The 35-kDa protease activity was detected as "streaks" on the 3D gel with a molecular weight of 35-kDa at pI between 2.9 and 4.0.

6-h postinduction insoluble AXOKINE® sample. The amount of insoluble sample loaded was 20 μg protein per gel. AXOKINE® was visible on the 2D gel as a large dark protein spot/streak located at 22-kDa and with a pI of 5.92. The 35-kDa protease was not visible on the 2D gel, as expected, since the concentration of the 35-kDa protease was below the detection limit of silver staining [9]. AXOKINE® was also visible on the 3D gel as a small, dark protein spot located at 22-kDa and with a pI of 5.92. The AXOKINE® protein spot appeared smaller on the 3D gels compared to the 2D gels for two reasons. During the required incubation step, proteins diffuse, which makes the spots appear lighter. The gelatin incorporated into the gel matrix increases the background level in the 3D gels, thus reducing the resolution of protein spots. The 3D gel detected the up-regulated proteases as clearing streaks (zones) on the 3D gel. The three prominent streaks have approximately the same molecular weights as the multiple bands observed on the SDS-GPAGE gels shown in Figure 2A. The molecular weights obtained on the SDS-GPAGE and 3D gels are only rough estimates, since nonreducing electrophoresis conditions are used to preserve protease activity. The pIs of the up-regulated proteases were low, ranging between approximately 2.9 and 4.0.

The 3D gels showed the protease activity as "streaks." These "streaks" of protease activity could be due to additional proteases or multiple forms of the 35-kDa protease. (Note: no inhibitors were added to the incubation buffer.) The "streaky" nature of the protease activity was most likely the result of incomplete focusing in the first dimension due to the nonreducing electrophoresis conditions required to observe protease activity after the separation. The location of the up-regulated proteases on the 3D gel corresponds to an area on the 2D gel where no proteins were detected (Figure 5).

Conclusions

An industry-wide challenge is the proteolytic degradation of recombinant proteins when produced in host organisms such as *E. coli*. The stringent and heat-shock responses are known to induce these proteases as well. The up-regulated proteases, due to recombinant AXOKINE® overexpression, upon IPTG induction, were detected and characterized. SDS-GPAGE analysis showed that the up-regulated protease activity was associated with both the soluble and insoluble fractions in *E. coli*. Western blot analysis determined that AXOKINE® in both the soluble and insoluble fractions was degraded as the fermentation progressed. Through the use of protease inhibitors, it was determined that the up-regulated protease (35-kDa) was not a serine or aspartic protease, but probably a methalloprotease.

PMSF, a serine protease inhibitor, significantly enhanced the protease activity of the up-regulated proteases. By varying the incubation buffer composition, it was determined that the 35-kDa protease was Mg^{2+} sensitive and ATP independent. Specifically, Mg^{2+} was required for optimal protease activity. A two-dimensional electrophoresis technique determined that the proteases had a low pI, between 2.9 and 4.0, and was possibly multimeric. The behavior of the up-regulated proteases was significantly different from the reported characteristics of OmpT. The molecular weight and ATP dependency of the up-regulated proteases were significantly different from the reported characteristics of La. In summary, this study indicated that there is at least one more unidentified *E. coli* protease that is up-regulated by recombinant protein overexpression.

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