

Original Article

A Sensitive Assay for Proteases in Bioaerosol Samples: Characterization and Quantification of Airborne Proteases in Salmon Industry Work Environments

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Abstract

Proteases are probably underestimated exposure agents in bioaerosols. Their roles as barrier disrupters in allergic sensitization and activators of innate inflammation call for more attention in exposure-response studies. The main objectives of this study was (i) to establish a suitable method for detection of small quantities of proteases in filtered air samples and (ii) to utilize the method to characterize exposure to proteases in a salmon industry work environment. Analysis of proteases in filtered air samples was based on zymography, using sodium dodecyl sulfate–polyacrylamide gel electrophoresis with 0.1% gelatin as substrate added in the polyacrylamide gel. Gelatinase activity was evident as cleared (unstained) regions. The area of these regions was quantified using image analysis (UVP Vision Works®). Standard curves with known amounts of active porcine trypsin were added to each gel. Validation of 11 non-linear standard curves showed R^2 (range) = 0.8989–0.9882, limit of detection = 0.056 nM, lower limit of quantification = 0.161 nM, and coefficients of variations (range) = 20–28%. Sampling of bioaerosols in salmon industry was performed using polytetrafluorethylene filters with an airflow of 3 l min⁻¹. All samples contained visible bands close to the size of porcine trypsin (23.3 kDa). The bands did not disappear in the presence of EDTA but abolished by Pefabloc, demonstrating that the enzyme is a serine protease, most likely salmon trypsin. Airborne levels of active protease were below the statistical detection limit in the filleting department but quantifiable in extract samples from the slaughter department. Three filtered air samples from the slaughter department showed air concentrations of 6.2, 16.5, and 27.0 ng m⁻³ air. We conclude that zymography is a sensitive and reliable method for exposure assessment of active proteases in indoor

environmental samples. We recommend this assay for use in occupational studies to characterize and quantify exposure to active proteases in bioaerosols.

Keywords: airborne proteases; bioaerosols; exposure assessment; exposure levels; occupational; salmon industry; seafood; trypsin; work environment; zymography

Introduction

Workers in seafood industry experience respiratory symptoms and illness associated with inhalation of aerosols containing biological material. Symptoms from upper and lower airways have been described among these workers (Gaddie et al., 1980; Sherson et al., 1989; Douglas et al., 1995; Bang et al., 2005a; Jeebhay and Cartier, 2010; Shiryayeva et al., 2010, 2014). The prevalence of occupational asthma associated with seafood exposure is estimated to be 2–36% in different workplaces [reviewed in Jeebhay and Cartier (2010)]. Both immunological (allergic) and non-immunological mechanisms are involved.

Bioaerosols are liberated to the air by mechanical processing of seafood or by air and water beams directed towards surfaces containing organic matter. To reveal the relative importance of various bioactive agents present in bioaerosols with respect to health effects, there is a need for exposure-response studies. However, lack of validated methods for exposure assessments of the small quantities of potential harmful agents that are present in ambient air at workplaces often limit the conclusions reached.

Enzymes as active agents in workplace bioaerosols are relevant in numerous work environments with the presence of organic matter of plant, animal, or microbial origin. In bakeries, the enzyme alpha amylase added in flour is important in the development of bakers' asthma (Wuthrich and Baur, 1990; De Zotti et al., 1994; Valdivieso et al., 1994; Salcedo et al., 2011). In detergent industry, various proteases and lipases are linked to asthma, allergy, and other hypersensitivity reactions among workers (Schweigert et al., 2000; Sarlo and Kirchner, 2002). Further, proteases in agricultural dust activate airway inflammation in mouse models (Romberger et al., 2015). In a previous study in crab industry, we have shown that the level of protease activity in bioaerosol samples varies by industrial process and factory-specific factors (Thomassen et al., 2016). Concentrations of active proteases in ambient air are generally low, challenging sampling and extraction procedures and analytic assays. Proteases are also known to potentiate the effect of other harmful agents in bioaerosols, acting as barrier disrupters in allergic sensitization (Ebeling et al., 2005; Asaduzzaman et al., 2015) and displaying a synergistic cooperativity with

endotoxins (Ostrowska et al., 2007; Rallabhandi et al., 2008; Bhagwat et al., 2015).

Many proteases act as sensitizers with the potential of eliciting an allergic response in the sensitized individual (Jacquet, 2011). However, also non-allergenic proteases are believed to play a role in inflammatory responses in airways (Reed, 2007). Studies, primarily on cellular models, have built up increasing evidence that exposure to external proteases, e.g. from mites, cockroaches and fungi, may cause barrier disruption and inflammation in the airways surface lining (Reed, 2007).

In our previous research, we have demonstrated that proteases extracted and purified from marine seafood are able to stimulate inflammatory signaling pathways in the airway epithelial cell model A549, via a specific mechanism involving protease-activated receptor-2 (PAR-2). Seafood proteases shown to display this ability include trypsin derived from salmon, sardine, and king crab (Larsen et al., 2008; Larsen et al., 2011; Bhagwat et al., 2014).

Epidemiological studies including quantitative exposure data are needed to confirm a possible link between inhaled proteases in bioaerosols and health outcomes in exposed persons. Quantification of proteases in bioaerosols is also relevant in workplace risk assessment protocols as basis for decision making on preventive measures or in public spaces where bioaerosols may be present as farm markets or near public waste deposits.

The most common analytical techniques for quantification of trypsin activity are based on hydrolysis of chromogenic substrates. The resulting liberation of a colored product can be measured spectrophotometrically. However, in environmental air samples, the levels of proteases are often below detection limit for these assays. In addition, poor stability at the temperatures normally found in production areas (Bang et al., 2005b) limits the use of long-term sampling to increase sample concentration. On this background, we explored zymography as a sensitive technique for assessing protease activity in environmental air samples. Zymography is a simple sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) technique enabling the detection of very low levels of protease activity, based on addition of substrate (e.g. gelatin) to the SDS–PAGE gel. Proteases are visualized as clear (unstained) bands against the

stained background (undigested substrate) (Kleiner and Stetler-Stevenson, 1994; Leber and Balkwill, 1997).

In this study, we present a zymographic assay optimized for detection of proteases <100 kDa, in bioaerosol samples collected from salmon industry work environments. Validation data for the zymographic assay is presented. In addition, we present procedures for sampling and extraction of bioaerosol samples containing enzymes, based on our experience from exposure studies in seafood industry. We report for the first time airborne concentrations of a protease at the size of trypsin, sampled in a Norwegian salmon factory workplace atmosphere.

Materials and methods

Reagents

Trypsin from Porcine pancreas (T-7409), *N* α -Benzoyl-d,l-arginine 4-nitroanilide hydrochloride (DL-BAPNA, B4875), Pefabloc, EDTA, acrylamide, Brij-35, and bromophenol blue were purchased from Sigma Aldrich (MO). Polytetrafluorethylene (PTFE) filters were purchased from SKC Ltd (UK). SDS and Coomassie brilliant blue were purchased from Merck (Germany).

Salmon industry work environment

Live, anesthetized salmon, typically 3–6 kg, are stunned and allowed to bleed out in large tanks. After automated gutting, visceral remains are removed manually and the fish may be decapitated, fileted, or packed directly. Environmental conditions are characterized by

low temperatures (e.g. <10°C) and high humidity (e.g. above 60% RH). Machineries, manual operations, and squirts from cleaning processes and water nozzels along conveyor belts release particles and droplets containing biological matter into the air.

Sampling and extraction of bioaerosol from salmon industry work environment

Figure 1 shows the equipment used for stationary sampling of bioaerosol in a salmon industry plant. PTFE filters, pore size of 1.0 μ (Millipore Billerica, USA), were placed into three-piece filter cassettes (3M, NO) and coupled to sampling pumps adjusted to produce an airflow of 3 l min⁻¹. The sampling pumps were placed in plastic boxes with the filters outside the box and mounted at workstations as close as reasonable practicable to the breathing zones of the workers. Samples were collected continuously for 10 h distributed over two following work shifts. After the sampling period, the filter cassettes were kept at –20°C for 1 day, transported in frozen condition to the lab, and transferred to –70°C until extraction. For the extraction procedure, the filters were removed from the filter cassettes with a pincer and eluted in 700- μ l phosphate-buffered saline (PBS) with 0.05% Tween 20 by rotation at 4°C over night. After extraction, the samples were aliquoted in smaller volumes and kept at –70°C.

Preparation of zymography gels

SDS polyacrylamide gels (7.5 cm $\times$ 8.5 cm $\times$ 0.75 mm) containing 0.1% (w/v) of gelatin were prepared.

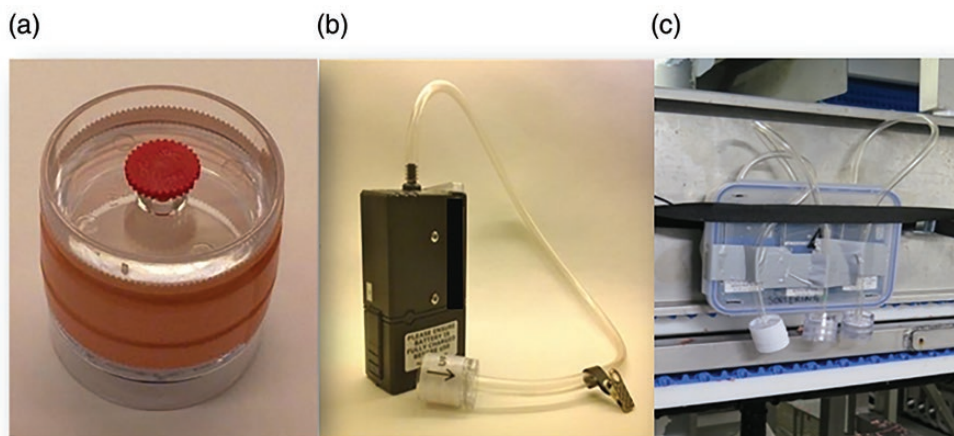


Figure 1. Equipment used for bioaerosol sampling in workplace air in salmon processing industry. PTFE filters were placed in three-piece filter cassettes (a) and coupled to sampling pumps (b) adjusted to produce an airflow of 3 L/min. For environmental sampling, the sampling pumps were placed in plastic boxes (c) with the filters placed outside the box and with sampling orifices headed downwards to protect from water squirts.

Stacking gels contained 5% (w/v) polyacrylamide in 0.064 M Tris, pH 6.8. Separating gels contained 13% (w/v) polyacrylamide in 0.374 M Tris-HCl, pH 8.8. Gels were polymerized by addition of 0.03% (w/v) ammonium persulfate (final concentration) and 0.1% TEMED.

Zymography assay

Samples to be analyzed were mixed with loading buffer [1 M Tris-HCl (pH 6.8), 10% (w/v) SDS, and 0.03% (w/v) bromophenol blue and 87% (v/v) glycerol] and loaded on the gel. Zymography was carried out as described previously (Malla et al., 2013). Briefly, the gel was subjected to electrophoresis at 20 mA/gel for 2 h. Thereafter, the gel was washed twice in 100-ml washing buffer (2.5% Triton X-100) followed by overnight incubation in developing buffer [50 mM Tris-HCl (pH 7.5), 5 mM CaCl_2 , 1 μM ZnCl_2 , 1 mM Brij-35] at 37°C. The gels were then stained in 0.2% Coomassie Brilliant Blue R-250 in 60% methanol, and the proteolytic activity of gelatin-degrading enzymes was detected as clear zones against the stained background.

Determination of protease subgroup

Filter extracts sampled in salmon industry work environment were applied on parallel gels, and the zymography assay was performed. To determine the protease class responsible for the observed gelatinolytic activity, the parallel gels were washed and incubated in buffer containing no additives (control), 10 mM EDTA (metalloprotease inhibitor), or 1.0 mM Pefabloc (serine protease inhibitor) before stained.

Enzyme activity analysis using spectrophotometry

The enzymatic activity of trypsin stock solutions was determined on a Perkin-Elmer λ -16 spectrometer coupled to a computer with the UV WinLab program, using the chromogenic substrate Benzoyl-d,l-arginine 4-nitroanilide hydrochloride (DL-BAPNA). The initial rate of the reaction was determined at 405 nm, 25°C using a total assay volume of 1.0 ml in a cuvette with a path length of 1.0 cm. The reaction mixture contained enzyme, DL-BAPNA, 10 mM CaCl_2 , and 7.5% DMSO in 25 mM Tris-HCl pH 8.1. K_m determination for porcine trypsin was carried out at the conditions described above, using ~200 nM of enzyme and 0.2–15.0 mM of DL-BAPNA. All assays were performed in at least triplicate. A rate assay was obtained using the above-described conditions with varying concentrations of porcine trypsin (25–700 nM) and a fixed concentration of 12 mM DL-BAPNA. The porcine trypsin was dissolved in 15 mM Glycin-HCl pH 3.0, and the amount of protein was determined at 280 nm using the extinction coefficient, $\epsilon_{280\text{nm}}^{1\%} = 15$. All assays were performed in at least triplicate.

Preparation of trypsin standards for quantitative zymography

Trypsin was dissolved in 15 mM Glycin-HCl, pH 3.0 giving a stock solution of ~74 μM . Small batches of this stock solution were stored at -20°C. To test the stability of trypsin during these storing conditions, aliquots were thawed at appropriate time points from the day of freezing up to Day 83. The enzyme activity at each time point was tested, using the spectrophotometric rate assay. Three parallels were performed at each time point. To obtain reliable and reproducible trypsin standard curves using the zymography assay, the concentration of active enzyme in stock solutions was determined by the spectrophotometric assay each day before diluted and used in the zymography assay.

Preparation of standard curves based on image analyses of gel pictures

Trypsin standards were mixed with loading buffer and loaded on the gel. After completion of the zymography assay, the gel was photographed in a BioDoc-It® Imaging system, UVP®. The intensity of trypsin bands was quantified using the software UVP Vision Works LS Image Acquisition and Analysis. I-max, describing maximal intensity, was chosen as the preferred quantification parameter. Standard curves were plotted as concentration of active porcine trypsin against I-max. As zero controls, we used two wells on each gel containing only loading buffer. The obtained intensity for the average of the zero controls was subtracted from the intensity of all samples and standards. A standard curve was drawn for each gel and the curves showed non-linear hyperbolic saturation as shown previously for other proteases (Kleiner and Stetler-Stevenson, 1994; Leber and Balkwill, 1997). As the curves were approximately linear only at the lowest trypsin concentrations, we used a non-linear equation to fit the best line to the obtained I-max data. The equation used was $y = y(\text{max}) \times (1 - e^{-(k \times x)})$, where y is the I-max intensity, $y(\text{max})$ is the maximal intensity, x is the concentration of trypsin in nM, and k a constant that is unique for each curve. The regression coefficient (r^2) shows how well the curve fits the determined I-max intensity values. The computer program used was GraphPad Prism 5.

Quantification of enzyme activity in bioaerosol samples

Filter extracts were analyzed using the zymography assay. Concentration of active enzyme in unknown samples was estimated from I-max values, using standard curves run at the same gel as described above. Higher concentration samples were diluted to fit into the

standard curve. Air concentrations were calculated from the concentrations in filter extracts, airflow through the filters, and sampling time. Airflow was controlled by a Defender 520M calibrator (Bios Int, USA) before and after sampling. Blank filters (FB) had been subjected to the same treatment as exposed filters and were extracted in the same way as these. The enzyme concentration of FB extracts was subtracted before calculating air concentrations.

Validation of the zymography assay for quantification of enzyme activity in filter extracts

Since the staining of the gel will vary between assays, we evaluated the method with respect to obtaining statistical significant differences between I-max-readings for different samples. I-max readings of zero controls, blank filter extracts, and extracts of filter sample from 17 different gels were compared. For each reading, the average reading for two zero controls on the same gel was subtracted. Box and Whisker plot and independent sample *t*-tests (SigmaPlot) was used in this analysis.

The concentration of active enzyme in filter extracts was calculated from standard curves run on the same gel. The same filter extracts were run on 11 different gels, each containing a separate standard curve. Using the 11 parallel datasets, the statistical derived limit of detection (LOD) was defined as the mean enzyme concentration for the blank sample (FB) plus 3 standard deviations. The limit of quantification (LOQ) was defined as the mean concentration of FB plus 10 standard deviations (Armbruster et al., 1994; Rahman et al., 2010). Precision is presented as coefficients of variation, CV% (SD/mean $\times$ 100), using results from three different samples having enzyme activities within the standard curve (S1, S2, and S3).

Statistical analyses

Statistical analyses were performed using Excel and Sigma Plot. Standard deviation (SD) and coefficient of variation (CV) were used as descriptive parameters in the validation of standard curves.

Results

Protease activity in extracts of filtered air from salmon industry work environment

Protease activity in extracts of air filters is shown in [Supplementary Fig. 1](#) (available at *Annals of Work Exposures and Health* online). Item A of the figure shows that all samples contain visible bands close to the size of porcine trypsin (23.3 kDa). The bands were not affected by the presence of EDTA in item B, but

abolished by Pefabloc in item C. This demonstrates that the band is not a metalloprotease but a serine protease, most likely salmon trypsin (Outzen et al., 1996). In addition, several proteins above 100 kDa with protease activity were present (not shown). On this background, we set out to develop a quantitative assay for protease activity in bioaerosol filter extracts.

K_m determination and rate assay

Figure 2A shows a Michaelis-Menten plot of trypsin degradation of DL-BAPNA, i.e. the initial velocity ($\Delta A_{405\text{nm}}/\text{min}$) against [DL-BAPNA] (mM). The results were analyzed with the program GraphPad Prism using the algorithm for a Michaelis-Menten equation. The K_m value was estimated to be 2.60 ± 0.05 mM with a regression coefficient r^2 equal to 0.998.

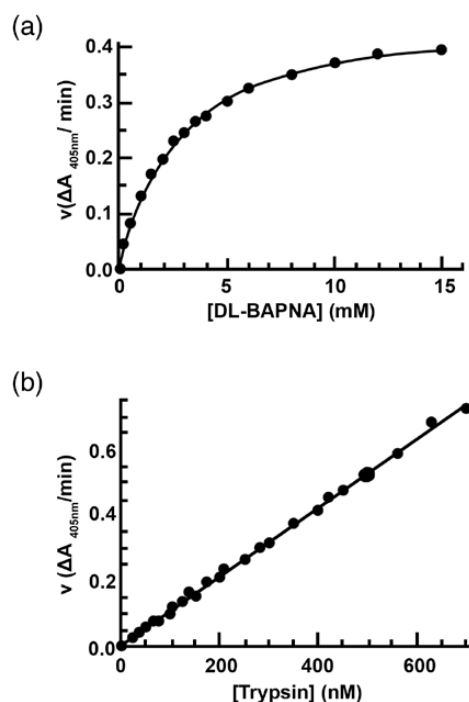


Figure 2. (a) Michaelis-Menten plot for porcine trypsin with DL-BAPNA as substrate. The reaction mixture contained 200 nM porcine trypsin, 0.2–15 mM DL-BAPNA, 10 mM CaCl_2 , and 7.5% DMSO in 25 mM Tris-HCl, pH 8.1. The initial rate ($\Delta A_{405\text{nm}}/\text{min}$) was determined as described in methods. Shown is the mean $\pm$ SD and each point represent at least three parallel measurements. (b) Enzymatic rate assay of porcine trypsin using DL-BAPNA as substrate. The rate assay shows the initial rate ($\Delta A_{405\text{nm}}/\text{min}$) vs the concentration of trypsin. The assay mixture contained 0–700 nM porcine trypsin, 12 mM DL-BAPNA, otherwise the same as in 2a. The initial velocity was determined as described in methods. Shown mean $\pm$ SD, each point represent at least three parallel measurements.

To obtain a reliable and reproducible rate assay for trypsin degradation of DL-BAPNA, highest possible substrate concentration should be used compared to its K_m value. Based on the substrates' solubility, cost, and the Michaelis-Menten plot (Fig. 2A), a [DL-BAPNA] of 12 mM was used in the rate assay. This ensured a minimum fluctuation of activity attributable to pipetting errors causing variable substrate concentrations and hence gave the most reproducible rate assay curve, i.e. initial velocity ($\Delta A_{405nm}/min$) against trypsin concentration. As shown in Fig. 2B, the rate assay plot is linear ($v = k [e]$) giving a rate constant k of $1.050 \pm 0.006 \Delta A_{405nm}/min/\mu M$ trypsin and a correlation coefficient r^2 of 0.9992. The rate constant k was used

to determine the amount of active trypsin in the frozen batches used for the zymography standard curves.

The stability of the frozen stock solution of trypsin was tested using the rate assay. This revealed that the enzyme was stable at these conditions for at least 83 days (see Supplementary Fig.2, available at *Annals of Work Exposures and Health* online).

Validation of the zymography assay

Figure 3 shows three representative zymographic gels and the corresponding standard curves.

As the curves are mainly linear only at the lowest trypsin concentrations, we chose to use a nonlinear function for the determination of enzyme

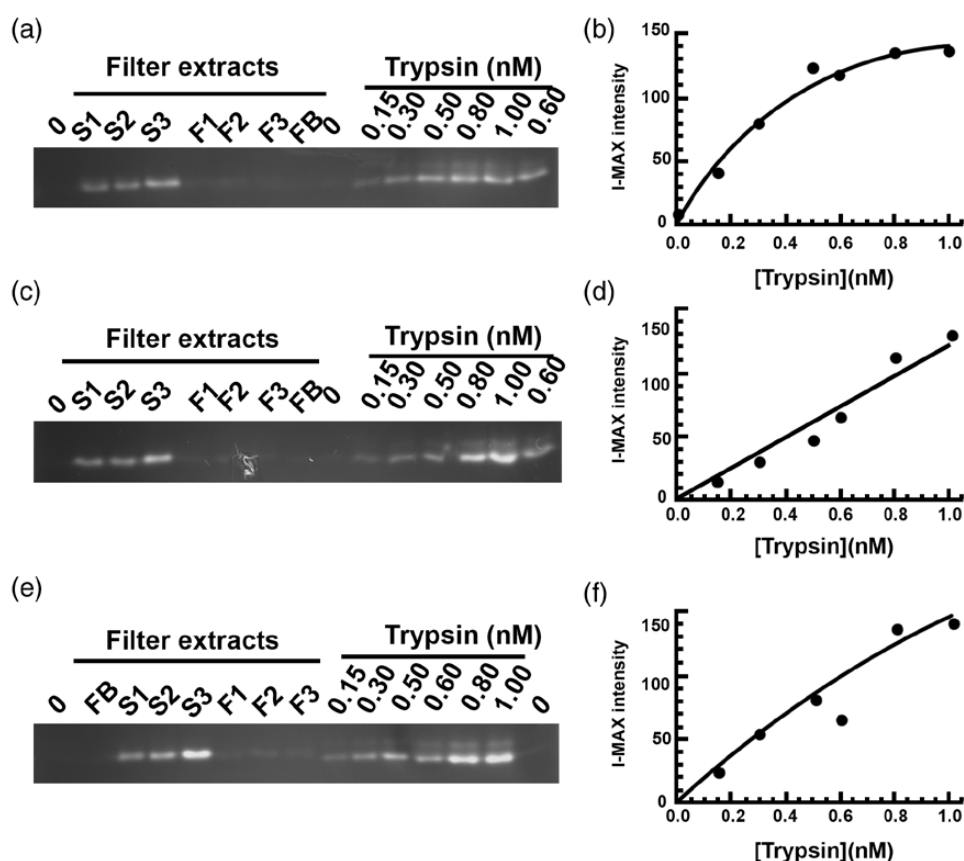


Figure 3. Three typical quantifications of trypsin like enzymes in filtered air samples from salmon industry work environments using gelatin zymography. Air samples from salmon industry work environments were collected on PTFE filters by means of a sampling pump and extracted as described in methods. S1-S3 and F1-F3 are extracts of filters placed at different locations the working environment as described in methods. FB (filter blank) is the extract from a clean empty filter. The amount of the trypsin standards in nM per well are shown above the gelatin zymography gels (a, c and e). The blank (0) contained just the loading buffer. (b, d and e) Shows the obtained standard curves used to determine the quantity of trypsin like enzymes in the air samples. The I-max intensity was determined using the BioDoc-It® Imaging system, UVP® as described in methods. The standard curves (I-max intensity vs [trypsin]) was determined with the GraphPad Prism 5 program, using the nonlinear equation: $y = y_{max} * (1 - e^{(-k * x)})$ where y is the I-max intensity, x is the concentration of trypsin in nM and k a constant that is unique for each curve. (▲) is a data point not included in the standard curve. The regression coefficients (r^2) for the three curves were: (b) 0.98, (d) 0.96 and (e) 0.99.

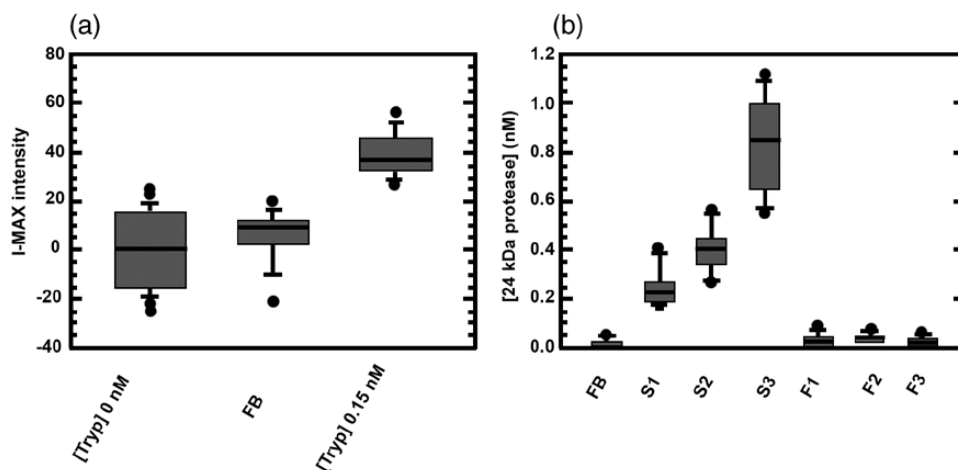


Figure 4. (a) Box and Whisker Plot showing the spread and median values ($n = 17$) of intensity readings (I-max) of zero controls, filter blanks (FB) and trypsin standard 0.15 nM with and without the inclusion of outliers. (b) Box and Whisker Plot showing the spread and median values ($n = 11$) of the concentration of the 24 kDa protease in the filter banks (FB) and industry samples from slaughter department (S1, S2, S3) and filleting department (F1, F2 and F3) estimated from the standard curves as described in the Method section.

concentrations in unknown samples. The non-linear regression gave less variable results for concentrations in the upper end of the standard curve. The lowest concentration of porcine trypsin producing a visible clear zone varied between gels. From the visual inspection of all the gels in preliminary test runs, 0.15 nM of trypsin was selected as the lowest standard. To evaluate the ability of the zymography assay to discriminate between controls and exposed samples, we analyzed the results from 17 gels containing trypsin standards (0.15–1.0 nM), zero controls (0 nM), and filter blanks (FB). The results revealed that the filter blanks were detected within the variation of the zero controls ($P = 0.393$) while the 0.15 nM trypsin control was well above the zero controls and the filter blanks ($P < 0.001$). The spread of the results and the median values after subtraction of zero controls are shown in the Box and Whisker Plot (Fig. 4A and B), and the mean $\pm$ SD I-max values were as follows: Zero controls (0 ± 16), FB (6 ± 10), and 0.15 nM trypsin (39 ± 9).

For further validation, we used 11 gels each containing complete standard curves of trypsin (0.15–1.0 nM) as shown in Fig. 3, zero controls, filter blanks, and extracts of exposed filter from salmon industry containing serine proteases at the size of trypsin in amounts between the lowest and highest trypsin standards. R^2 of standard curves were in the range 0.8989–0.9892, with median 0.9785. The LOD was 0.056 nM and limit of quantification (LOQ) 0.161 nM. CVs were between 20 and 28% for the detectable samples.

Quantification of protease activity in extracts of filtered air from salmon industry work environment

Figure 3A, C and E shows that the filter extracts contained a protease at the size of trypsin.

After preliminary experiments showing high enzyme activity, samples S1 and S2 were diluted 7.5 and 6 times, respectively, before applied to the gel, while the other samples were undiluted.

Visually it was not possible to see any gelatin degradation in the filter blank (FB) samples. They were identical to the zero control (i.e. the loading buffer). However, in some of the gels, the imaging system detected a small difference from the zero control intensity readings, corresponding to trypsin concentrations ranging from 0 to 0.05 nM (mean $\pm$ SD of 0.011 ± 0.015 nM; Table 1). The estimated FB concentration was not statistically significant from the zero control. We evaluated the obtained values for the collected air samples against the filter blank (FB). Three air samples, F1, F2 and F3, gave faint bands in some of the gels and were invisible in other. The estimated amounts varied from undetectable (0 nM) to 0.073 nM. Thus, also the highest detectable levels were below the LOQ. Table 1 shows the obtained mean $\pm$ SD for these samples, and Fig. 4B shows the Box and Whisker plot. The values are not significantly increased compared to the filter blank.

The two diluted samples S1 and S2 resulted in bands that were in the lower range of the standard curve and their enzyme concentrations calculated from the

Table 1. Concentrations of trypsin-like enzyme in ambient air at workstations in a salmon factory. Samples of bioaerosols were collected on PTFE filters coupled to a sampling pump. Extracts of the filter samples were diluted if necessary and analyzed using the zymography assay as described. The levels given below are mean levels and standard deviations based on results from 11 different zymographic gels. FB, filter blank; S1, S2, and S3 are filtered air samples from slaughter department; F1, F2, and F3 are filtered air samples from filleting department.

Sample no.	Sampling site	Active enzyme concentration in filter extract, mean $\pm$ SD (nM)	Enzyme concentration in ambient air ^a , mean $\pm$ SD (ng m ⁻³)
FB		0.011 $\pm$ 0.015	ND
S1	Scraping ^b	1.846 $\pm$ 0.525 ^c	26.97 $\pm$ 8.03
S2	Sorting	2.200 $\pm$ 0.864 ^d	16.50 $\pm$ 6.68
S3	Slaughtering	0.829 $\pm$ 0.177	6.22 $\pm$ 1.35
F1	Conveyer belt	0.029 $\pm$ 0.027	ND
F2	Filleting machine	0.032 $\pm$ 0.020	ND
F3	Conveyer belt	0.021 $\pm$ 0.017	ND

ND, not determined.

^aCorrected for filter blanks (FB).

^bScraping off entrails residues from degutted fish.

^cValue is corrected for 7.5 $\times$ dilution.

^dValue is corrected for 6 $\times$ dilution.

standard curve, varied from 0.18 to 0.40 nM and 0.27 to 0.56 nM for S1 and S2 (Fig. 4B), with mean $\pm$ SD levels of 0.25 $\pm$ 0.07 and 0.40 $\pm$ 0.08 nM, respectively. This fitted good with the visual evaluation of the zymography gels. They were both statistically significantly higher than the filter blank ($P < 0.001$). The undiluted sample S3 had a band intensity that corresponded to trypsin concentrations in the upper range of the standard curve. The determined value ranged from 0.6 to 1.1 nM (Fig. 4B) with mean value $\pm$ SD of 0.82 $\pm$ 0.17 nM. This value was significantly larger than the values for the two diluted samples S1 (0.25 $\pm$ 0.07 nM) and S2 (0.40 $\pm$ 0.08 nM), $P < 0.001$. Similarly, the value for S2 was significantly larger than the value for S1, $P < 0.001$. All this shows that it is also possible to statistically distinguish samples with different activity localized within the standard curve used.

Table 1 presents the enzyme concentrations (mean $\pm$ SD) in the filter extracts and the air concentrations of enzyme at workstations in the slaughter department of a salmon industry plant. The calculated air concentration was significantly different from each other ($P < 0.01$). Air concentrations in the fileting department were not assessed since the filter extracts were not distinguishable from the filter blank samples.

Table 1 shows that the extract concentration of the 24 kDa protease was below the statistical detection limit of 0.056 nM in the filleting department but above the LOQ (0.16 nM) for all samples in the slaughtering department of the investigated factory. Calculation of coefficients of variation gave precision values between 20 and 28% for the samples within the quantification range.

Discussion

This study shows that zymography is a well-suited method to determine the concentration of active proteases in workplace samples of bioaerosols. In addition, molecular weight estimation based on migration distance in the zymographic gel in comparison with known molecular weight proteins, combined with enzyme inhibition profiling and/or antibody-based detection, allows characterization and provides certain identification of many proteases. The method is relatively simple, low cost, and requires commonly used technical equipment, making it within reach of most commercial and academic laboratories engaged in analysis of environmental samples. Sensitivity of protease detection can be increased by increasing the incubation time and/or incubation temperature. Down to femtomole quantities of proteases have been detected using this technique (Li et al., 2010). However, as the time of incubation increases, the proteases may diffuse within the gel. Close bands may then merge, reducing the chance to detect all proteolytic enzymes in the sample.

Gelatin zymography is an activity-based method, as the detection principle is directly linked to enzyme activity, measuring the transparency of non-stained gel bands where gelatin has been digested by the protease. As gel qualities, incubation conditions, and staining may differ between each analysis, standards with known protease activities need to be present on each gel. To prepare standards for the zymography assay, it is necessary to determine enzyme activity of the stock solution each day the zymography assay is performed. This is due to

the lowered activity of solubilized trypsin over time, even when stored frozen at -20°C (Supplementary Fig. 2, available at *Annals of Work Exposures and Health* online). For this purpose, a rate assay was determined, relating enzyme activity to molar concentration (Fig. 2B).

In detergent industry, airborne protease activity was first quantified using activity-based assays based on chromogenic or fluorogenic substrates, which are enzymatically converted to release colored or fluorescent products (Bruce, 1978). However, an important limitation of these assays for measuring airborne concentration is sensitivity. Fluorogenic assays are often more sensitive than colorimetric assays, but non-specific fluorescence quenching activity limits their usefulness with crude samples. Immunological based assays have also been used in detergent industry (Miller, 1994). Mass spectrometry (MS) and PCR-based techniques are powerful techniques that may be utilized also for proteases. However, these tools rely on extensive molecular data prior to analysis, restricting the applicability for poorly characterized mixtures as many environmental sample extracts. Specialized technical skills among personnel and high-cost instrumentation also limit the use in smaller laboratories. Moreover, these techniques do not consider enzyme activity as they only detect the presence and possibly quantity of the protein.

Sampling in seafood industry needs special consideration of frequent water squirts and a high water content of the ambient air (Bang et al., 2005b). The filters should have hydrophobic qualities to avoid damage of filter structure by high humidity. A downwards pointing inlet is advantageous for squirt protection. The polytetrafluoroethylene filters contained in three-piece filter cassettes from 3M were well suited for these conditions. PVC filters are cheaper hydrophobic filters that could be good alternatives. A thorough testing of different combinations of filters and sampling heads should be performed with the aim of recommending the best suited equipment for sampling in these wet environments.

Rapid freezing of the filters is essential to stop possible degradation and impairment of enzyme activity. For the same reason, the sampling time could not be extended to several days, as is common for some chemical exposure agents. Thus, we chose to limit the sampling time to 10 h to ensure a detectable level of protease in most samples and at the same time avoid extensive impairment of enzyme activity during sampling. Low amounts of enzymes in air samples and risk of activity impairment during extraction also need to be considered in the extraction procedure. An extraction volume of 700 μl and an extraction time of 4 h were chosen to meet both these challenges. Up-concentration of extracts

was attempted using a ProteoSpin™ Detergent Clean-up Micro Kit (Norgen Biotek Corp.). However, the procedure gave large variation in enzyme recovery (data not shown). Increasing the extraction time over night gave no further increase in enzyme activity (data not shown).

The r^2 values of 11 zymographic standard curves were in the range 0.8989 to 0.9892, with mean and median values of 0.9656 and 0.9785, confirming that the transparency of the gel band is directly proportional to the concentration of active enzyme. A statistical detection limit of 0.056 nM and a quantification limit of 0.161 nM show that the method is highly sensitive and able to distinguish enzyme activity from background noise at levels far below the limits of most chromogenic and fluorogenic assays. The precision, presented as CV, was 20–28% in our hands. A CV <10 % is often required for assays used in public service or commercial laboratories. However, for many biological-based assays, CVs between 20 and 50% are common. Variations in structural qualities and coloration of the zymographic gel are probably the most important contributors to result variability. The CV of the assay should be considered in result analyses and interpretation. Using a semiquantification approach is a rational way of dealing with low precision data and should be considered when using active enzyme concentrations as exposure parameter in epidemiological studies. We have previously used a three-level semiquantification scale based on the 25th and 75th percentile of protease activity in an occupational exposure study in Norwegian crab industry (Thomassen et al., 2016).

Conclusion

Gelatin zymography is a sensitive and reliable method for exposure assessment of active proteases in indoor environmental samples. We recommend this assay for use in occupational studies to characterize and quantify exposure to active proteases in bioaerosols.

Supplementary data

Supplementary data are available at *Annals of Work Exposures and Health* online.

Declaration for Publication

The authors declare no conflict of interest relating to the material presented in this article. Its contents, including any opinions and/or conclusions expressed, are solely those of the authors. The author(s) is/are/were employed by the University Hospital North Norway, Uit The Arctic University of Norway who

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Online Supplementary Material

A sensitive assay for proteases in bioaerosol samples. Characterization and quantification of airborne proteases in salmon industry work environments.

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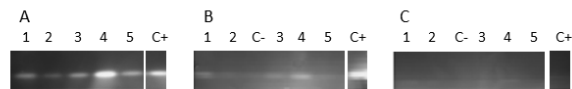
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Supplementary figure 1.

Zymography of filtered air samples from salmon industry work environments and porcine trypsin as molecular weight marker. Air samples from salmon industry work environments were collected on PTFE filters by means of a sampling pump and extracted as described in the methods. **A**: Filtered air samples, reference gel. **B**: Same samples as in A, but the wash and developing buffer also contained 10 mM EDTA. **C**: Same samples as in A treated with 1mM Pefabloc. 1-5: samples from work stations in salmon industry, C+: positive control porcine trypsin 23.3 kDa, C-: Negative control empty lane

Supplementary figure 1



Supplementary figure 2.

Enzyme activity of trypsin stock solution (approximately 74 μM) after variable storing time up to 83 days at - 20°C.

Supplementary Figure S2

