Vaccine 37 (2019) 6518–6525

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

# Ultraviolet irradiation of trypsin, lysozyme and β-galactosidase: how does UVC affect these enzymes when used as a secondary barrier against adventitious agents?

Michelle A. Gabriel [a,b], Emma V. Dare [a], Sarah M. Meunier [a], J. Larry Campbell [c], Michael R. Sasges [b], Marc G. Aucoin [a,*]

[a] Department of Chemical Engineering, University of Waterloo, Waterloo, Ontario N2L 3G1, Canada
[b] Trojan Technologies, London, Ontario, Canada
[c] SCIEX, Concord, Ontario, Canada

ARTICLE INFO

*Article history:*
Received 27 June 2019
Received in revised form 15 August 2019
Accepted 26 August 2019
Available online 10 September 2019

*Keywords:*
Ultraviolet light
Irradiation
Trypsin
Disinfection

ABSTRACT

Trypsin is one of the essential raw materials used in the manufacturing of biopharmaceutical products. As an animal derived product, it can potentially carry a serious risk of contamination with adventitious agents that can result in production shut down and lost product. To mitigate these risks, several methods are currently being used in the industry to remove contamination including physical and chemical methods. Ultraviolet-C (UVC) light is known to inactivate adventitious agents that are resistant to physical and chemical methods and could be a secondary barrier strategy. In this study, we investigated the effect of UVC irradiation on the activity and structure of trypsin. Extreme doses of UVC light were applied to trypsin using a collimated beam apparatus. The effect of UVC light on trypsin enzymatic activity was measured using a colorimetric activity assay and the effect on structure was analyzed by spectrophotometry, gel electrophoresis, and mass spectrometry. To broaden the scope, the effect of UVC light on the activity of two additional enzymes, lysozyme and β-galactosidase, was also examined. At high doses of UVC light, changes to protein structure and protein fragmentation resulted in decreased trypsin activity. However, minimal damage was observed at doses applicable to inactivating adventitious agents, making UVC a feasible treatment for viral inactivation of trypsin products.



## 1. Introduction

Contamination is a persistent challenge that has been faced by the biopharmaceutical industry over the past 25 years, and continues to be a serious issue [1–10]. A recent survey determined that batch failures occur every 51.3 weeks, roughly once every year, and the primary cause of these failures is contamination [11]. Viral or bacterial contamination can by very costly and lead to complete shutdown of production and biological product supply shortage. Contamination can also result in altered cell growth or altered properties of the biological product being produced. Most notably, the much publicized vesivirus 2117 contamination event at Genzyme in 2009 is estimated to have cost $1 billion [12,13]. In addition, patients went months without their medication as this contamination event was rectified.

Many of the more recent cases of contamination have been associated with raw materials, suggesting that animal-derived products may be one of the main portals of viral and microbial entry to biopharmaceutical manufacturing environments. For example, porcine circovirus (PCV) was identified in a live attenuated rotavirus vaccine, Rotarix™, manufactured by GlaxoSmithKline in 2010 [9,14]. It was later confirmed that this viral contamination originated from porcine trypsin. PCV is not the only virus to be concerned about. Fifty-five porcine virus species from 17 different families have the potential to contaminate porcine trypsin and impact the health of cells in culture [15].

Trypsin (EC 3.4.21.4) is an animal-derived material extracted from the pancreatic gland of pigs and inherently carries a high risk of contamination. Trypsin has several applications during both upstream and downstream biological product manufacturing. The main use of trypsin in upstream processing is the detachment of cells from culture container walls during growth and passaging. In downstream processing, trypsin can be used in product processing, vaccine activation, and as a protein-cleaving reagent [16].

* Corresponding author.
*E-mail address:* marc.aucoin@uwaterloo.ca (M.G. Aucoin).

https://doi.org/10.1016/j.vaccine.2019.08.063
0264-410X/

To mitigate the high risk in animal-derived materials including trypsin, ultraviolet-C (UVC) treatment is being considered as a barrier against contamination by bacteria and viruses of solutions of proteins and enzymes. For example, recent guidance from the European Medicines Agency (EMA) recommends the use of two complementary virus reduction steps for trypsin, including a final inactivation step of gamma, e-beam, or UV irradiation [17]. Biopharmaceutical manufacturers Genzyme and Sanofi Pasteur also support UV inactivation as a promising technology to reduce the risk of contamination from raw materials [18]. UV irradiation is currently used globally to effectively disinfect drinking and waste water [19]. UV inactivation is achieved using mercury lamps emitting at 254 nm, in the UVC range. This wavelength overlaps with the absorption peak of nucleic acids and result in nucleic acid damage, which inactivates the adventitious agent by preventing microbial and viral replication. However, this wavelength also partially overlaps with the absorption of aromatic residues in proteins and can result in photo-degradation of proteins. Tryptophan, tyrosine, and phenylalanine residues absorb UVC light, with peak absorbance at 280 nm or 250 nm. Histidine has a peak absorbance at 215 nm at physiological pH, and does not directly absorb UVC light. Instead, histidine can be indirectly modified through the formation of singlet oxygen during UVC exposure [20,21].

In this study, we examine the effect of UVC light on trypsin. We also examine the effect of UVC light on two additional enzymes, lysozyme (EC 3.2.1.17) and β-galactosidase (EC 3.2.1.23), as surrogates to test the effect on a broader range of enzymes. Proteins were irradiated in batches using a collimated beam apparatus, and their function analyzed by activity assays, spectrophotometry, gel electrophoresis, and mass spectrometry.

## 2. Materials and methods

### 2.1. Collimated beam irradiation of enzymes

A 6.4 mg/mL stock solution of bovine trypsin (92% pure based on UV absorbance, Sigma Aldrich, Oakville, ON, Canada) was diluted to 10 μg/mL and 100 μg/mL in 66 mM Tris-HCl pH 8.1, 12 mM $CaCl_2$. 5 mL samples of dilute trypsin solution in 10 mL beakers were irradiated using a calibrated collimated beam apparatus (Trojan Technologies, London, ON, Canada) incorporating a low-pressure mercury lamp emitting at 254 nm [22]. Beakers were placed in an ice water bath with continuous stirring to avoid thermal degradation during irradiation. Optical absorbance of the fluid was measured with a Cary 100 spectrophotometer (Agilent, USA) equipped with a 6-inch integrating sphere. Corrections for optical absorbance and other factors were included [22], and samples were continuously stirred and exposed to UV light to achieve average doses of 50, 100, 200, 500, and 1000 mJ/cm$^2$. Control samples were also placed in an ice water bath, but were not exposed to UV light. The trypsin enzyme activity was determined using a colorimetric Trypsin Assay Kit (Abcam, Toronto, ON, Canada) as per the manufacturer's instructions. Each sample was measured in triplicate.

For lysozyme, a 0.026 mg/mL and 0.304 mg/mL chicken lysozyme solution was prepared in lysozyme assay buffer (66 mM potassium phosphate pH 6.24). After irradiation, lysozyme activity was determined using a colorimetric Lysozyme Assay Kit (Sigma-Aldrich, Oakville, ON, Canada) as per the manufacturer's instructions. Each sample was measured in triplicate.

For β -galactosidase, stock β-galactosidase enzyme solution (Sigma-Aldrich, Oakville, ON, Canada) was diluted 1:10 000 and 1:1000 in lysis buffer (50 mM HEPES pH 7.5, 5 mM CHAPS). After irradiation, β-galactosidase activity was determined using a colorimetric Galactosidase Assay Kit (Sigma-Aldrich, Oakville, ON, Canada) as per the manufacturer's instructions. Each sample was measured in triplicate.

### 2.2. Spectrophotometric analysis

0.1 mg/mL irradiated trypsin solutions were transferred to a 1 cm quartz cuvette and the absorbance measured from 200 to 800 nm with a Cary50 spectrophotometer (Agilent, USA). To measure the number of reduced cysteine residues, Ellman's reagent was used [23,24]. The absorbance of a 0.2 mM 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB) (Sigma-Aldrich, Oakville, ON, Canada), 2.5 mM sodium acetate in 100 mM Tris-HCl pH 8.0 solution was measured in a 1 cm quartz cuvette at 412 nm with a Cary50 spectrophotometer (Agilent, USA). 15 μL of 0.1 mg/mL irradiated trypsin was added, mixed, incubated at room temperature for 5 min, and the absorbance due to the formation of 2-nitro-5-thiobenzoate (TNB) measured at 412 nm. The protein was unfolded by the addition of 5 M urea and the assay repeated. The concentration of free thiol was calculated using N-acetyl-L-cysteine (Sigma-Aldrich, Oakville, ON, Canada) as a standard. Each sample was measured in triplicate.

### 2.3. Gel electrophoresis

5 μg of trypsin, from 0.1 mg/mL trypsin solutions that were irradiated, were diluted with Laemmli sample buffer (2% SDS, 10% glycerol, 0.02% bromophenol blue in 125 mM Tris-HCl pH 6.8) with and without reducing agent (355 mM β-mercaptoethanol) and boiled at 95° for 5 min. Samples were separated by 18% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for 2 h at 150 V in SDS/Tris/glycine running buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.1% SDS) using the Mini Protean II gel apparatus (Bio-Rad, Burlington, ON, Canada) [25]. Gels were stained with Coomassie (0.1% Coomassie Blue R250 in 10% acetic acid, 50% methanol) and destained (10% acetic acid, 50% methanol). Densitometry of the bands was performed using ImageJ [26].

### 2.4. Liquid chromatography mass spectrometry (LC-MS)

The LC-MS experiments were performed using a Shimadzu (Kyoto, Japan) Prominence UFLC coupled to an X500B quadrupole time-of-flight (QTOF) mass spectrometer (SCIEX, Concord, ON, Canada). The analytical LC column used was a Phenomenex (Torrance, CA, USA) Kinetex C8, with a particle size of 2.6 μm, pore size of 100 Å and a dimension of 2.1x50mm. The mobile phase was composed of A (water:acetonitrile 98:2 (v/v) + 0.1% formic acid) and B (acetonitrile:water 98:2 (v/v) + 0.1% formic acid). The trypsin samples were diluted 10-fold from their original stock and 5 μL of this sample was injected on-column. To elute the trypsin and any UV-treatment by-products from the LC column, a linear solvent gradient that ramped the mobile phase from 5% B to 95% B in 6 min was employed; the 95% B level was maintained for 30 s, after which the mobile phase was returned to 5% B for 10 s. The % B level was cycled to 95% four times in rapid succession (i.e., 5% to 95% B in 15 s) to clean the LC column of any residual material that failed to elute during the initial, longer gradient. Solvent eluting from the LC column was subjected to electrospray ionization (ESI) by the QTOF's ESI source that was maintained at a voltage of 5500 V with the source temperature at 400 °C. Nitrogen was used as the curtain gas (30 psi), the ion source nebulization (GS1 at 50 psi) and desolvation (GS2 at 50 psi) gases. Mass spectra were recorded in positive-ion mode from $m/z$ 600–4000. LC-MS data were analyzed using BioPharmaView™ 2.0 and PeakView (research-grade) software 1.2.2.0 (64-bit) (SCIEX). Mass/charge data were extracted from the LC chromatogram in seven time frames (0.3–0.6,

0.6–1.0, 1.0–2.2, 2.2–3.2, 3.2–4.0, 4.0–4.6, 4.6–6.2 min). Peaks that changed with UV dose were quantified using the area.

## 3. Results

UVC exposure can cause modifications of aromatic and cystine (cysteine involved in a disulfide bond) residues that lead to structural changes, which can affect protein function. Proteins strongly absorb UV light around 280 nm due to the presence of aromatic residues (tryptophan, tyrosine, and phenylalanine) and disulfide bonds.

Trypsin is a protein classified as a serine protease that cleaves protein backbones at the carboxyl side of lysine or arginine amino acids, except when followed by a proline [27]. Active trypsin is 223 amino acids in length, and contains four tryptophan, ten tyrosine, three histidine, and three phenylalanine residues. As seen from the space-filled structure of trypsin, only a fraction of these residues are on the surface of the protein (Fig. 1a and b). The remaining aromatic residues are buried within the core of the enzyme. Only one of the four tryptophan residues, and seven of the ten tyrosine residues are on the surface of trypsin and directly exposed to the exterior under normal conditions. The secondary structure is ß-strand rich and the tertiary structure is stabilized by six disulfide bonds between twelve cysteine residues (Fig. 1c). The active site contains a highly conserved Histidine-Aspartate-Serine catalytic triad (highlighted in green in Fig. 1c).

### 3.1. UVC irradiation of trypsin

To determine the effect of UVC light on trypsin activity, trypsin solutions at two concentrations were irradiated using a collimated beam apparatus and the activity measured by cleavage of a colorimetric substrate (Fig. 2). The trypsin activity decreased with increasing applied dose as compared to a non-irradiated trypsin control (Fig. 2a). The decrease in trypsin activity with UV dose appears to be first-order, with an inactivation rate of 0.0007 percent activity/mJ/cm$^2$. At a dose of 100 mJ/cm$^2$, the trypsin retained 93–96% activity, not different from the activity of the control. At the most extreme dose applied, trypsin activity decreased by 50%. However, there was no observed difference between the two concentrations; the trypsin activity appears to be a direct function of dose, independent of concentration. The trypsin activity also decreases in a first-order manner with absorbed energy, but the decrease in trypsin activity per unit absorbed energy is dependent on trypsin concentration (Fig. 2b). More energy is required to decrease the activity of trypsin at 0.1 mg/mL than at 0.01 mg/mL.

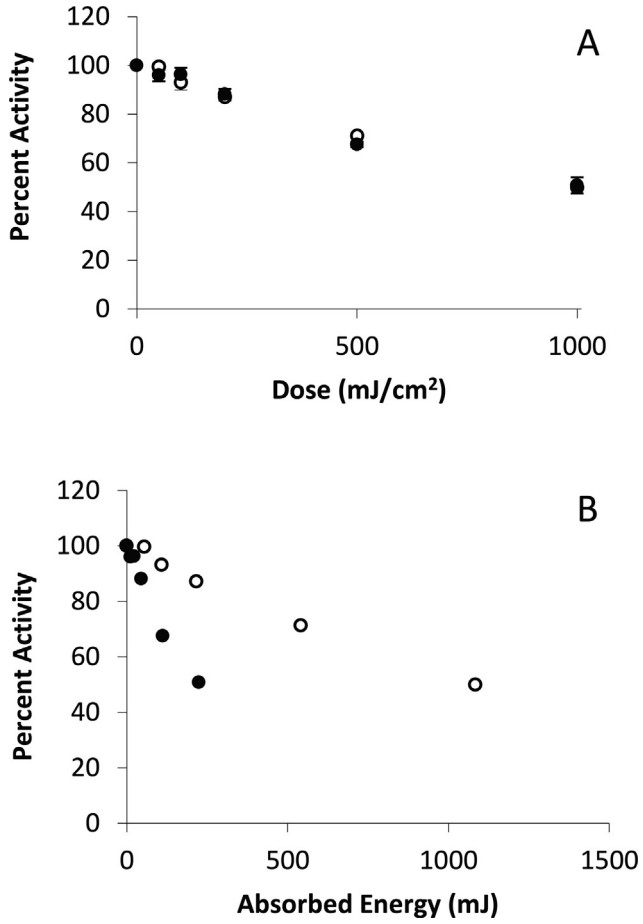


**Fig. 2.** Exposure to UVC light decreases the enzymatic activity of trypsin. Trypsin activity was plotted as a function of applied dose (A) and absorbed energy (B) for 0.01 mg/mL (closed circle) and 0.1 mg/mL (open circle) trypsin.

The amount of UV light is reported as both absorbed energy and dose. Absorbed energy is the amount of energy absorbed by a fluid and relates to the first law of photochemistry, which states that light must be absorbed by a chemical substance in order for a photochemical reaction to take place. More relevant for disinfection is UV dose (properly denoted as fluence). Dose is the total amount of direction-independent UV irradiance incident on the fluid multiplied by time. To illustrate these two terms, take the two solutions of increasing trypsin concentration. As the trypsin concentration

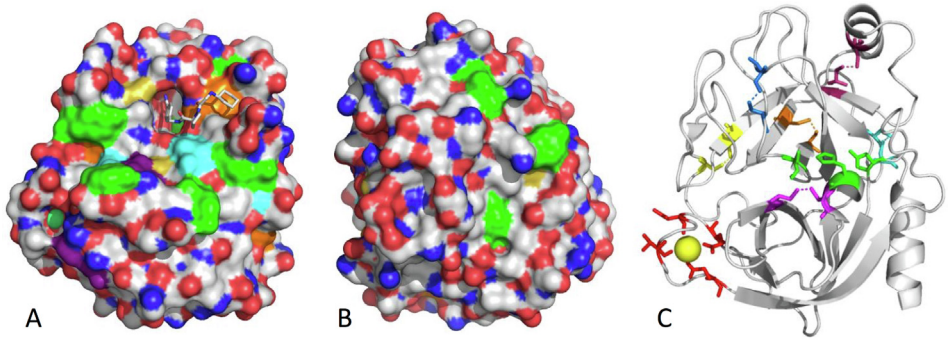


**Fig. 1.** The structure of trypsin protein. 180° representation of the surface of trypsin (A and B). Surface exposed tryptophan, tyrosine, histidine, and phenylalanine residues are highlighted in orange, green, cyan, and purple, respectively. A peptide inhibitor is shown in the active site pocket. C) Ribbon diagram of trypsin. The active site residues are shown in green. Residues coordinating the calcium (shown as yellow ball) binding pocket are shown in red. Twelve cysteine residues and their disulfide bonds are also shown (blue, yellow, orange, pink, magenta, turquoise). Images prepared with Pymol using PDB 2ZQ1 structure [28]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

increases, the fluid becomes more opaque to 254 nm light and the average flux decreases. To deliver the same dose to a high concentration (high absorbance) sample, a longer exposure time is required. The absorbed energy, conversely, increases as the fluid becomes more opaque. The higher concentration sample will have higher absorbed energy than the lower concentration sample, because the absorbed energy per unit time is higher and the exposure time is also higher.

In an effort to see if there are any changes to the structure of trypsin, the UV/Vis spectra of irradiated trypsin was measured (Fig. 3a). Trypsin has two absorption peaks. The strong absorption peak at 200 nm is due to the peptide backbone of the protein. The peak at 280 nm is due to the presence of aromatic residues. The resultant absorbance is dependent on the micro-environment surrounding the aromatic residue and as a consequence, residues that are surface-exposed and residues that are buried within the protein absorb differently. Increasing doses of UV resulted in an increase in absorbance of trypsin at 279 nm as well as at 250 nm. In addition, the shoulders of the peak have become broader as the UV dose increased. These changes suggest that changes have occurred to the environment surrounding aromatic residues and disulfide bonds. Hydrophobic aromatic residues absorb at 280 nm and are typically found buried in the hydrophobic core of proteins. Residues buried on the inside of the protein are shielded from absorbing light. Increases in absorbance at 280 nm suggest that aromatic residues are absorbing light and may be indicative of a weakened hydrophobic core in the protein or buried aromatic residues becoming surface-exposed. The broadening of the shoulders above 300 nm could be UV-induced modifications of tryptophan residues, namely kynurenine and N-formylkynurenine (NFK), which absorb at longer wavelengths [29]. UV altered the native conformation of trypsin and these alterations were dose-dependent.

The structure of trypsin is stabilized by six disulfide bonds. These bonds are sensitive to breakage by UV light. To investigate the reduction of disulfide bonds to cysteines, free thiols present in irradiated trypsin were reacted with DTNB and the formation of TNB was measured at 412 nm (Fig. 3b). Minimal incorporation of TNB was detected in the folded conformation of trypsin, although there might be an increasing trend with dose. The addition of urea after UV irradiation results in an unfolded conformation of trypsin and an increase in the incorporation of TNB. This suggests that trypsin maintains a folded conformation at high doses of UV, since the free cysteines were unable to react with DTNB in the absence of urea. With urea, the incorporation increases to about 4 mol/mol trypsin after a dose of 100 mJ/cm$^2$ and then slowly decreases to about 1.5 mol/mol trypsin with increasing UV dose. Four mol TNB/mol trypsin is equivalent to four free thiols, or the breakage of two disulfide bonds to yield four cysteine residues per trypsin molecule, on average. The reduction of cysteine residues at higher UV doses could be due to the formation new disulfide bonds or UV-induced modifications of the free thiol groups.

Protein fragmentation and aggregation can also occur due to exposure to UVC light. Trypsin samples treated with UVC were analyzed by gel electrophoresis (Fig. 3c). Under reducing condi-

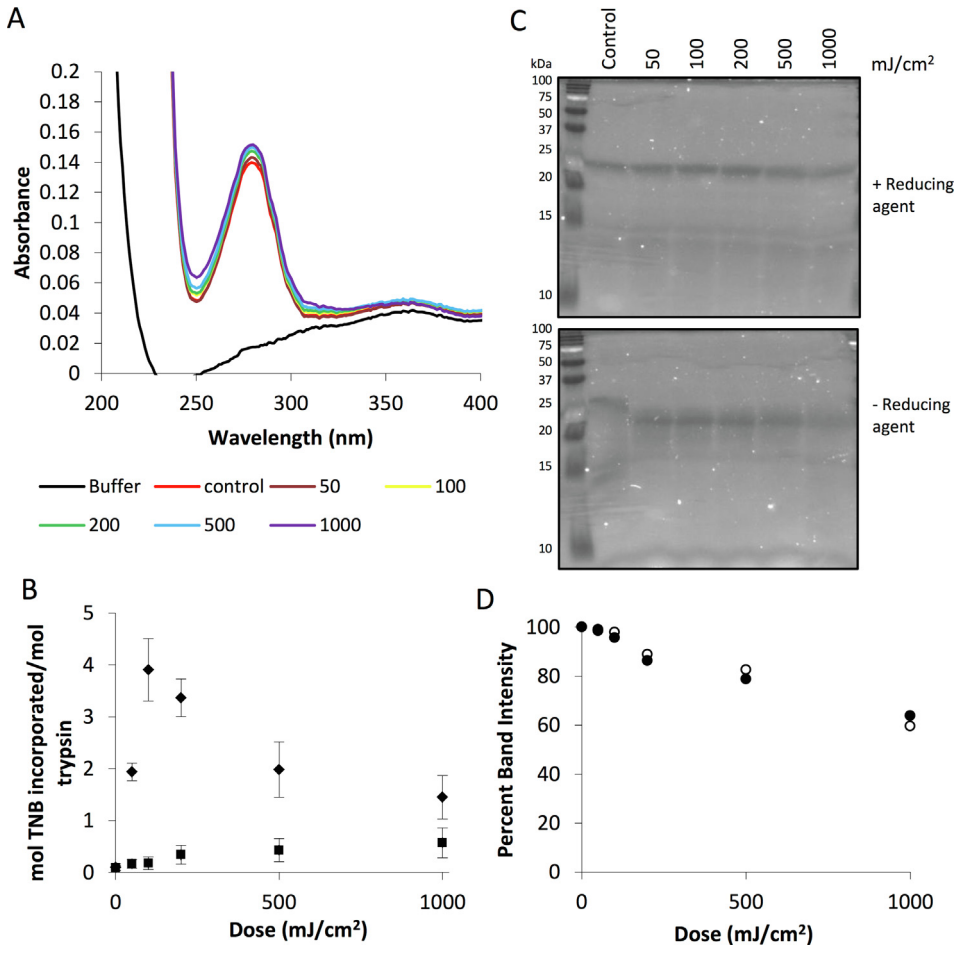


**Fig. 3.** Structural changes in trypsin are induced by exposure to UVC light. A) The absorbance spectra of irradiated 0.1 mg/mL trypsin. B) The incorporation of TNB by free thiols in irradiated trypsin in the native (square) and unfolded with 5 M urea (diamond) state. C) SDS-PAGE analysis of irradiated trypsin with and without B-mercaptoethanol reducing agent and D) the percent band intensity for trypsin as quantified by dosimetry with (closed circle) and (open circle) without reducing agent.

tions (β-mercaptoethanol), all disulfide bonds are reduced. As expected, trypsin migrates to a mass of about 23 kDa in the control and irradiated samples. Under non-reducing conditions, disulfide bonds are not reduced. The control sample migrates larger than expected suggesting that the disulfide bonds are present and slowing the migration. The irradiated samples, even as low as 50 mJ/cm$^2$, have a band migrating lower than the non-irradiated control and at the same mass as the reduced samples. This suggests that some disulfide bonds in the irradiated samples are no longer intact. Bands of larger molecular weights were not observed in either reducing or non-reducing conditions suggesting that the trypsin protein has not aggregated through covalent or disulfide bonds. Quantification of these bands determined that the amount of intact trypsin is decreasing with increasing UVC dose, suggesting that trypsin is being fragmented (Fig. 3d). However, no bands of smaller molecular mass, <23 kDa, were detectable with Coomassie staining. A dose of 100 mJ/cm$^2$ resulted in a slight reduction in intact protein, 95–97% of the control.

To further investigate UVC-induced fragmentation of trypsin, a more sensitive technique was employed. UVC treated trypsin samples were analyzed by LC-MS (Fig. 4). LC analysis of trypsin resolved two predominant peaks (Fig. 4a). The first peak at 0.4 min did not change with UVC dose, whereas intact trypsin has a retention time (RT) of 4.15–4.19 min and decreases in intensity with UVC dose. Several other smaller peaks with RT 0.7, 1.8, and 3.6 min appear with increasing UVC dose, indicating the formation of smaller peptides. There was no aggregation of trypsin observed, as no peptides were eluted with RT longer than intact trypsin. Based on these peaks, the LC chromatogram was broken into seven time frames and the mass spectra analyzed (Fig. 4b). The intensity of peptides that change with UVC dose was quantified for each time frame (Fig. 4c). With increasing UVC exposure, there is a decrease in the intact trypsin and a consequent increase in smaller peptide fragments. UV-cleavage products, 11–18 kDa (RT 4–4.6 min), increase in intensity up to doses of 200–500 mJ/cm$^2$ and then decrease with higher doses of UVC. Most likely, these cleavage products are being further fragmented with higher doses. While some small fragments, <10 kDa (RT 0–0.6 min), are present in the no UVC control and increase in intensity with higher doses of UVC, the majority of fragments appear only after doses greater than 200 mJ/cm$^2$ (RT 0.6–4.0 min). This may coincide with the doses required to fragment the large UV-cleavage products. At a dose of 100 mJ/cm$^2$, 20% of trypsin is degraded. At an extreme dose of 1000 mJ/cm$^2$, 65% of trypsin is degraded. The sequences of these peptide fragments as well as any UVC- induced modifications are currently unknown.

### 3.2. UVC irradiation of lysozyme

To determine the effect of UVC light on lysozyme activity, lysozyme at two concentrations was irradiated using a collimated beam apparatus and its activity measured (Fig. 5). Lysozyme activity decreased with increasing dose as compared to a non-irradiated control protein (Fig. 5a). At a dose of 100 mJ/cm$^2$, lysozyme activity was found to be not different from the activity of the control sample. The more concentrated lysozyme solution was more strongly affected by the same UVC dose as the lower concentration solution. The more concentrated solution is more opaque, requiring longer irradiation time to achieve the same dose, and deposits more energy into the sample. Lysozyme activity decreased with increasing absorbed energy and was similar between both lysozyme concentrations (Fig. 5b).

### 3.3. UVC irradiation of β -galactosidase

The activity of β -galactosidase decreased with increasing UVC dose as compared to a non-irradiated control protein (Fig. 6). At a dose of 100 mJ/cm$^2$, lysozyme activity was about 80% of the non-irradiated sample (Fig. 6a). However, there was no observed difference between the two concentrations; change in β-galactosidase activity appears to be a direct function of dose, independent of concentration. β-galactosidase activity decreased with absorbed energy, but the decrease in activity does not track with absorbed energy (Fig. 6b). This suggests that the photochemistry of the degradation is not a zero-order process.

## 4. Discussion

Recent cases of contamination by animal-derived products in biopharmaceutical processing continue to emphasize the importance of using multiple strategies to help mitigate the risk. In this study we investigated the effect of UVC irradiation on three enzymes, trypsin, lysozyme, and β-galactosidase. UVC-induced photo-oxidation of enzymes can occur by a direct or indirect mechanism. Direct photo-oxidation involves the absorbance of UVC light by the amino acid side chains of aromatic residues (tryptophan, tyrosine, and phenylalanine) and cystine. Indirect photo-oxidation occurs via radicals, reactive oxygen species, and reactive photo-products. Many protein and amino acid modifications occur as a result of UVC light exposure, and include disulfide bond breakage, cross-linking, deamination, oxidation, aggregation, and fragmentation. Collectively, these modifications could result in negative structural and functional effects on the enzyme.

Trypsin is a protease of animal origin frequently used in both upstream and downstream biopharmaceutical processes. When exposed to high doses of UVC light, trypsin activity is impaired but not completely diminished. Porcine trypsin treated with UVC doses of 200 mJ/cm$^2$ has been shown to be functionally equivalent to non-treated trypsin in the passaging of MRC-5 cells [30]. We have extended this to extreme doses of UVC to demonstrate that trypsin enzymatic activity decreases with increasing UVC dose which can be largely attributed to fragmentation of the intact protein, although modifications of aromatic residues and breakage of disulfide bonds may also occur.

Fragmentation of trypsin occurs as a result of small alterations in the structure of the protein in response to UVC, as partial unfolding of the tertiary structure of the protein could be exposing buried aromatic and cysteine residues and destabilizing the protein (Fig. 3ab). UVC results in the breakage of disulfide bonds to yield four cysteines that become chemically modified or reform new disulfide bonds (Fig. 3). Intermolecular disulfide bonds are not evident, as protein aggregation was not observed (Fig. 3c and Fig. 4a). Disulfide bonds can directly absorb UVC energy and indirectly via UVC excitation of aromatic residues that trigger the electron ejection from side chains which can be captured by a disulfide bond. Either way, the result is a neutral cysteine, reactive thiol radical that can reform new disulfide bonds, or conversion to oxoalanine. Structurally, these disulfide bonds serve to stabilize the tertiary structure of trypsin. Specifically, the cys191-cys220 disulfide bond (Fig. 1c, highlighted in blue) stabilizes the S1 pocket which is required for enzyme selectivity [31]. Trypsin cleaves peptide bonds after arginine and lysine, amino acids with long and positively charged side chains. The S1 pocket contains an aspartate that is negatively charged which attracts and stabilizes the positively charged side chains of arginine and lysine in the substrate [32]. Mutation of these two cysteines to alanines, which prevents disulfide bond formation, resulted in decreased ability to stabilize the transition state during enzyme catalysis, and reduced activity in recombinant rat trypsin expressed in yeast [31]. UV-induced breakage of this disulfide could have a similar effect on enzyme activity. To further support the photo-damage of disulfide bond by UV, Burke and Augenstein observed that cystine residues were

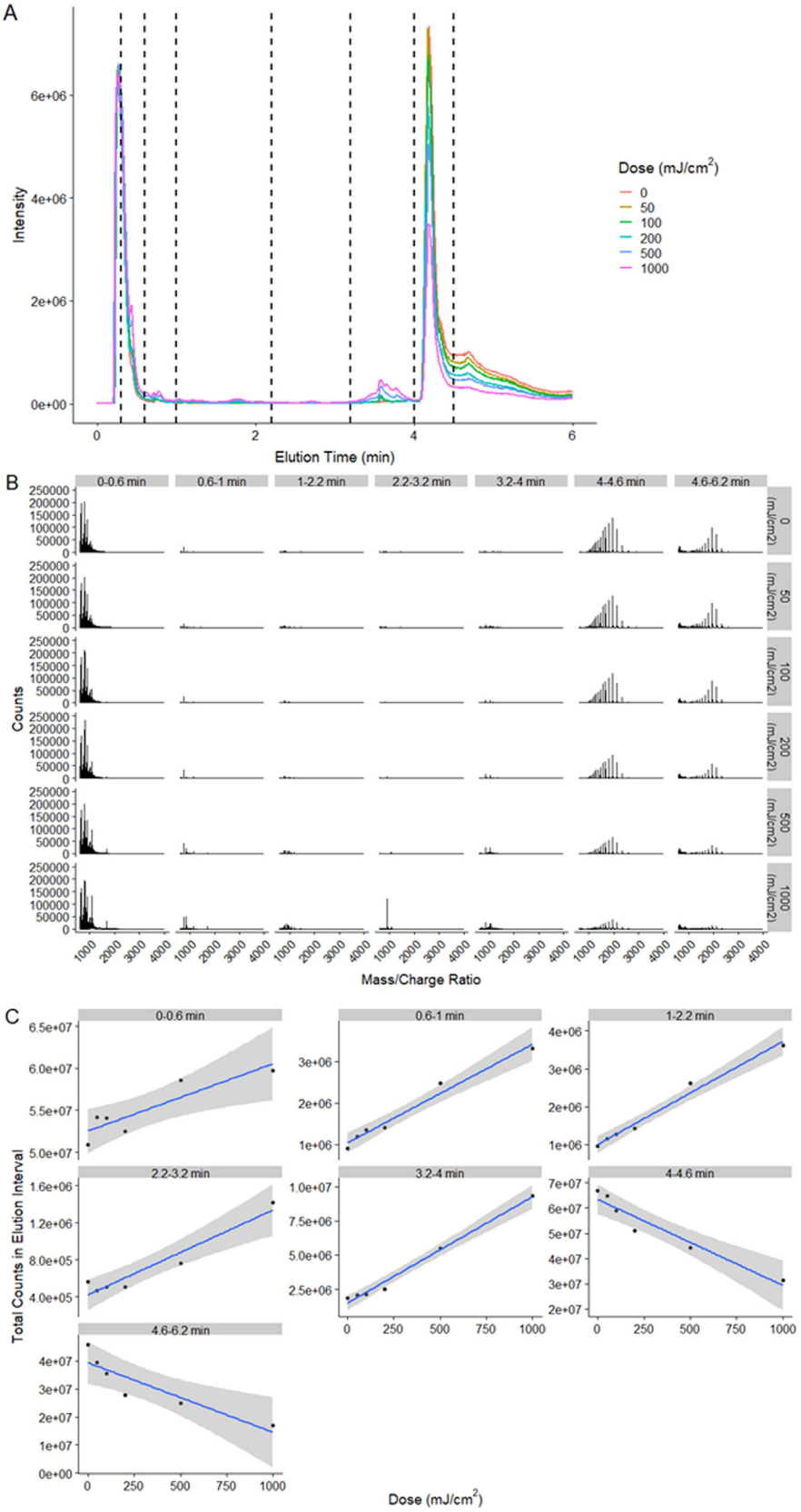


**Fig. 4.** LC-MS analysis of irradiated trypsin. A) LC chromatogram of irradiated trypsin protein. Vertical bars illustrate the seven time frames (in minutes) chosen for analysis. B) The intensity of peptides separated by MS at each dose for each time frame. C) Peaks that changed in intensity with UV dose were quantified using the area and plotted for each time frame.

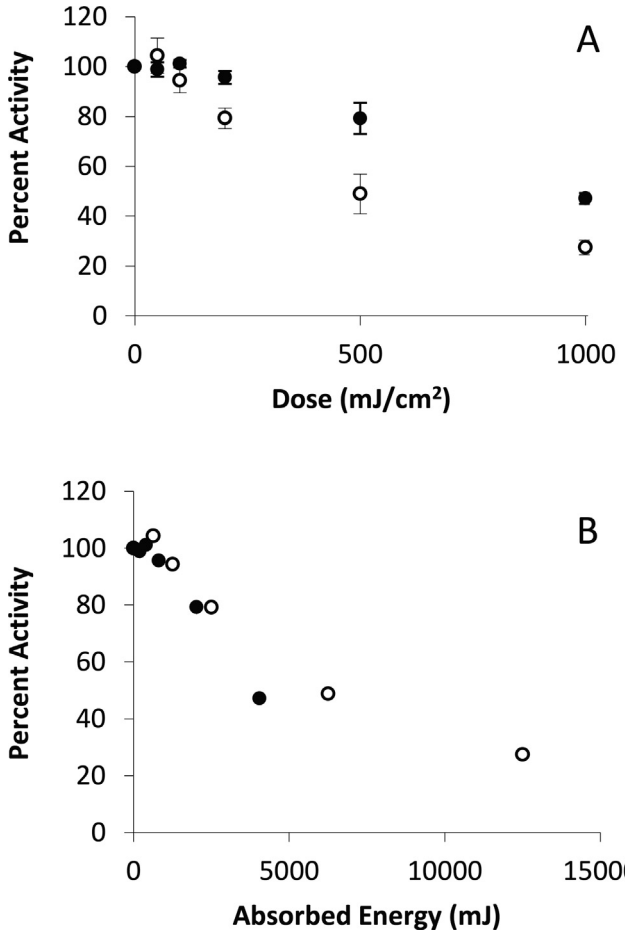


**Fig. 5.** Exposure to UVC light decreases the enzymatic activity of lysozyme. Trypsin activity was plotted as a function of applied dose (A) and absorbed energy (B) for 0.026 mg/mL (closed circle) and 0.304 mg/mL (open circle) lysozyme.

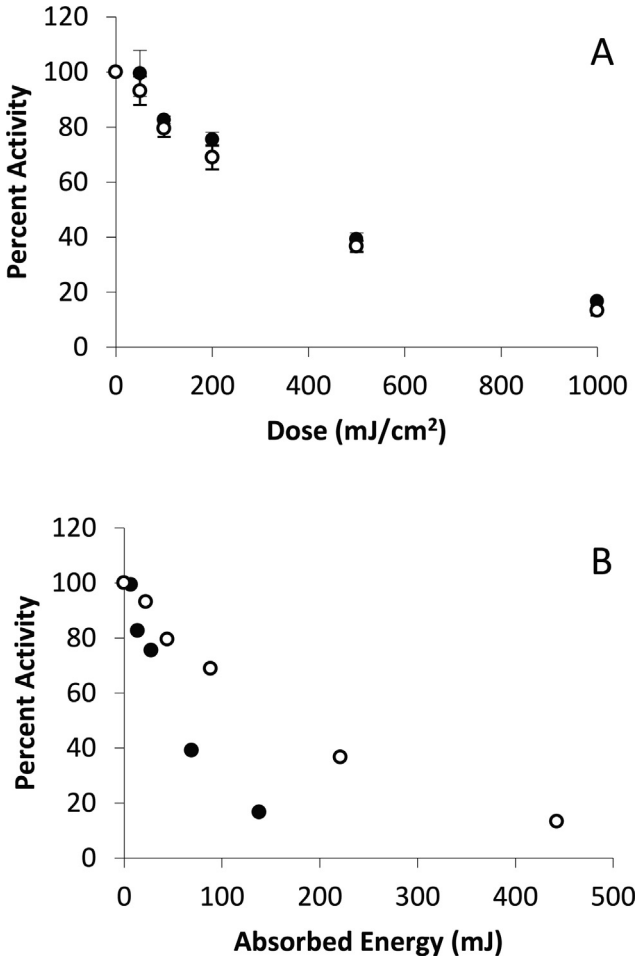


**Fig. 6.** Exposure to UVC light decreases the enzymatic activity of β-galactosidase. Trypsin activity was plotted as a function of applied dose (A) and absorbed energy (B) for 1:10 000 dilution (closed circle) and 1:1000 dilution (open circle) β-galactosidase.

the only amino acids affected by irradiation of dried bovine trypsin by UVC light and degradation occurred at the same rate as loss of enzymatic activity [33]. There was no evident loss of aromatic residues, suggesting no formation of photo-products kynurenine and NFK. Further LC-MS and amino acid analysis of irradiated trypsin will be necessary to identify hotspots for UVC-induced modifications and fragmentation.

In general, UVC irradiation caused a decrease in enzyme activity. This study applied high UVC doses intended to cause significant enzyme damage so that the effect could be studied. At lower doses sufficient to inactivate many adventitious agents, the decrease in enzyme activity was small, making UVC a feasible treatment for some enzymes and some target organisms [34]. The degree of inactivation of enzyme activity was not consistent across the three enzymes studied here. Trypsin was found to be relatively resistant to UVC irradiation, while β-galactosidase was strongly degraded by UVC irradiation (Figs. 2 and 6). Lysozyme had an intermediate sensitivity (Fig. 5). The tertiary structures of both trypsin and lysozyme are stabilized by disulfide bonds, whereas β-galactosidase is large a homotetramer not bound by disulfide bonds. Photo-activation of tryptophan groups in hen egg white lysozyme partially reduce disulfide bonds resulting in aggregation and structural changes [35]. Perhaps this partly explains the increased sensitivity of β-galactosidase to UVC. In addition, the UVC-induced damage was not always directly proportional to absorbed energy. This effect is surprising, suggesting that the mode of UVC-induced damage is complex and not described by a simple kinetic model.

For porcine trypsin, the EMA indicates that there are two particular viruses of concern: PCV and porcine parvovirus (PPV) [17]. These are small, single stranded DNA, non-enveloped viruses. Parvoviruses are 20–25 nm, while circoviruses are 17–20 nm. A small difference in size, but enough that filtration is not effective for the removal of circoviruses [36]. In addition, these viruses are resistant to inactivation approaches such as gamma irradiation and heating, chemical treatments such as solvents, detergents, and low pH, and are not inactivated by the enzymatic activity of trypsin [36–39]. DNA from these viruses has been detected in cell culture, trypsin, and a rotavirus vaccine [15,40,41]. Ultraviolet irradiation would be an independent inactivation method, relying on a completely different mode of action from filtration or chemical methods.

PPV is very sensitive to UVC, requiring only 2 mJ/cm$^2$ to achieve 1-log inactivation [42,43]. PCV is more resistant to UVC, requiring about 18 mJ/cm$^2$/log [43,44]. In order to achieve a 4-log inactivation credit for both organisms, a dose of about 80 mJ/cm$^2$ would be required. In the present study, a UVC dose of 100 mJ/cm$^2$ resulted in a trypsin activity about 93–96% of the control enzyme solution (Fig. 2). Based on the passaging of MRC-5 cells, it is expected that at this dose, irradiated trypsin will be functionally equivalent [30]. This could make UV a reasonable method for addressing PPV and PCV in trypsin, theoretically achieving a >12-log credit for PPV and

a 5-log credit for PCV at a dose of 100 mJ/cm$^2$. For gamma irradiation of dried trypsin, a dose of 50 kGy is required for 3-log inactivation of PPV [36,39,45]. This corresponds to about a 15% decrease in bovine or porcine trypsin activity [33,45,46]. However, PCV-2 is more resistant to gamma irradiation and requires a dose of 45 kGy to achieve 1-log inactivation [37]. A dose of 225 kGy would be required to achieve 5-log-clearance which would reduce the activity of trypsin by about 70% [33,46].

In summary, UVC irradiation of these enzymes caused a decrease in enzyme activity. However, minimal damage was observed at doses applicable to inactivating adventitious agents, making UV a feasible treatment for some enzymes and some target organisms.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

This work was supported in part by the generous contribution of SCIEX in the form of LC-MS, PeakView and BioPharmaView software. This work was made possible by a MITACS Elevate Postdoctoral Fellowship to MAG and an NSERC Collaborative Research and Development grant [grant number CRDPJ-484833-2015] to MGA.

## Contributions

MAG, MRS, and MGA conceptualized the study. MAG, EVD, and JLC collected the data. MAG, SMM and MGA analyzed the data. MAG wrote the manuscript. All authors reviewed the final version of the manuscript.

## References

[1] Drexler HG, Uphoff CC. Mycoplasma contamination of cell cultures: incidence, sources, effects, detection, elimination, prevention. Cytotechnology 2002;39(2):75–90.
[2] Pastoret PP. Human and animal vaccine contaminations. Biologicals 2010;38(3):332–4.
[3] Aranha H. Virus safety of biopharmaceuticals: absence of evidence is not evidence of absence. Contract Pharma 2011;13:82–7.
[4] Baylis SA, Finsterbusch T, Bannert N, Blümel J, Mankertz A. Analysis of porcine circovirus type 1 detected in Rotarix vaccine. Vaccine 2011;29(4):690–7.
[5] Moody M, Alves W, Varghese J, Khan F. Mouse Minute Virus (MMV) contamination–A case study: detection, root cause determination, and corrective actions. PDA J Pharm Sci Technol 2011;65(6):580–8.
[6] Skrine J. A biotech production facility contamination case study – mouse minute virus. PDA J Pharm Sci Technol 2011;65:599–611.
[7] Chen J, Bergevin J, Kiss R, Walker G, Battistoni T, Lufburrow P, et al. Case study: a novel bacterial contamination in cell culture production—Leptospira licerasiae. PDA J Pharm Sci Technol 2012;66(6):580–91.
[8] Qiu Y, Jones N, Busch M, Pan P, Keegan J, Zhou W, et al. Identification and quantitation of Vesivirus 2117 particles in bioreactor fluids from infected Chinese hamster ovary cell cultures. Biotechnol Bioeng 2013;110(5):1342–53.
[9] Petricciani J, Sheets R, Griffiths E, Knezevic I. Adventitious agents in viral vaccines: lessons learned from 4 case studies. Biologicals 2014;42(5):223–36.
[10] Saltzman J. Sanofi Genzyme issues recall for contaminated arthritis gel. Boston Globe; 2017. https://www.bostonglobe.com/business/2017/12/11/sanofi-genzyme-issues-recall-for-contaminated-arthritis-gel/ruFBNfmYSyJdMy9i7qwF8N/story.html Accessed on January 15, 2018.
[11] Langer ES. Average batch failure rate worsens. Genetic Eng Biotechnol News 2016;36(17).
[12] Ainsworth E, Weisman R. Virus shuts down Genzyme plant, holds up drugs for 8000. Boston Globe 2009.
[13] Rockoff JD. Drug manufacturing mending after questions of quality. Wall Street J 2010;11.
[14] Victoria JG, Wang C, Jones MS, Jaing C, McLoughlin K, Gardner S, et al. Viral nucleic acids in live-attenuated vaccines: detection of minority variants and an adventitious virus. J Virol. 2010;84(12):6033–40.
[15] Marcus-Sekura C, Richardson JC, Harston RK, Sane N, Sheets RL. Evaluation of the human host range of bovine and porcine viruses that may contaminate bovine serum and porcine trypsin used in the manufacture of biological products. Biologicals 2011;39:359–69.
[16] Prazeres DMF. Considerations on the use of enzymes in the downstream processing of biopharmeceuticals. Pharm Bioprocess 2016;4(5):91–5.
[17] European Medicines Agency (2015) Guideline on the Use of Porcine Trypsin Used in the Manufacture of Human Biological Medicinal Products. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2014/02/WC500162147.pdf.
[18] Goetschalckx S, Fabre V, Wynants M, Bertaux L, Plasvic M, Boussif O, et al. A holistic biosafety risk mitigation approach. Amer Pharm Rev 2014;17(4):48–56.
[19] Masschelein WJ, Rice RG. Ultraviolet light in water and wastewater sanitation. Boca Raton, FL, USA: CRC Press; 2002.
[20] Amano M, Kobayashi N, Yabuta M, Uchiyama S, Fukui K. Detection of histidine oxidation in a monoclonal immunoglobulin gamma (IgG) 1 antibody. Anal Chem 2014;86(15):7536–43.
[21] Marques EF, Medeiros MHG, Di Mascio P. Lysozyme oxidation by singlet molecular oxygen: Peptide characterization using [(18) O]-labeling oxygen and nLC-MS/MS. J Mass Spectrom 2017;52(11):739–51.
[22] Bolton JRJ, Linden KG. Standardization of methods for fluence (UV dose) determination in bench-scale UV experiments. J Environ Eng 2003;129:209–15.
[23] Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959;82(1):70–7.
[24] Riener CK, Kada G, Hermann JG. Quick measurement of protein sulfhydryls with Ellman's reagent and with 4,4′-dithiodipyridine. Anal Bioanal Chem 2002;373(4–5):266–76.
[25] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
[26] Rasband, WS, ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2019.
[27] Di Cera E. Serine proteases. IUBMB Life 2009;61(5):510–5.
[28] Brandt T, Holzmann N, Muley L, Khayat M, Wegscheid-Gerlach C, Baum B, et al. Congeneric but still distinct: how closely related trypsin ligands exhibit different thermodynamic and structural properties. J Mol Biol 2011;405(5):1170–87.
[29] Fukunaga Y, Katsuragi Y, Izumi T, Sakiyama F. Fluorescence characteristics of kynurenine and N′-formylkynurenine. Their use as reporters of the environment of tryptophan 62 in hen egg-white lysozyme. J Biochem 1982;92(1):129–41.
[30] Vaidya V, Dhere R, Agnihotri S, Muley R, Patil S, Pawar A. Ultraviolet-C irradiation for inactivation of viruses in foetal bovine serum. Vaccine 2018;36:4215–21.
[31] Wang EC, Hung SH, Cahoon M, Hedstrom L. The role of the Cys191-Cys220 disulfide bond in trypsin: new targets for engineering substrate specificity. Protein Eng 1997;10(4):405–11.
[32] Ma W, Tang C, Lai L. Specificity of trypsin and chymotrypsin: loop-motion-controlled dynamic correlation as a determinant. Biophys J 2005;89(2):1183–93.
[33] Burke M, Augenstein L. A comparison of the effects of ultraviolet and ionizing radiations on trypsin activity and on its constituent amino acids. Biochem J 1969;114(3):535–45.
[34] Meunier SM, Sasges M, Aucoin MG. Evaluating ultraviolet sensitivity of adventitious agents in biopharmaceutical manufacturing. J Ind Microbiol Biotechnol 2017;44:893–909.
[35] Wu LZ, Sheng YB, Xie JB, Wang W. Photoexcitation of tryptophan groups induced reduction of disulfide bonds in hen egg white lysozyme. J Mol Struct 2008;882:101–6.
[36] Nims R, Plavsic M. Identification of worst-case model viruses for selected viral clearance steps. BioProcess J 2014;13:6–13.
[37] Plavsic ZM, Bolin S. Resistance of porcine circovirus to gamma irradiation. BioPharm 2001;14(4):32–6.
[38] Welch J, Bienek C, Gomperts E, Simmonds P. Resistance of porcine circovirus and chicken anemia virus to virus inactivation procedures used for blood products. Transfusion 2006;46(11):1951–8.
[39] Nims RW, Gauvin G, Plavsic M. Gamma irradiation of animal sera for inactivation of viruses and mollicutes–a review. Biologicals 2011;39(6):370–7.
[40] Pinheiro de Oliveira TF, Fonseca Jr AA, Camargos MF, de Oliveira AM, Pinto Cottorello AC, Souza Ados R, et al. Detection of contaminants in cell cultures, sera and trypsin. Biologicals 2013;41(6):407–14.
[41] Pinheiro de Oliveira TF, Fonseca Júnior AA, Camargos MF, de Oliveira AM, Lima NF, Freitas ME, et al. Porcine parvovirus as a contaminant in cell cultures and laboratory supplies. Biologicals 2016;44(2):53–9.
[42] Wang J, Mauser A, Chao SF, Remington K, Treckmann R, Kaiser K, et al. Virus inactivation and protein recovery in a novel ultraviolet-C reactor. Vox Sang 2004;86(4):230–8.
[43] Lackner C, Leydold SM, Modrof J, Farcet MR, Grillberger L, Schäfer B, et al. Reduction of spiked porcine circovirus during the manufacture of a vero cell-derived vaccine. Vaccine 2014;32(18):2056–61.
[44] Remington K. Use of porcine circovirus as a challenge for filtration and other virus risk mitigation steps. 17th Planova Workshop, Washington, DC, June 12-13, 2014; 2014.
[45] Purtle DR, Festen RM, Etchberger KJ, Caffrey MB, Doak JA, Validated gamma radiated serum products. Research report R013. SAFC Biosciences; 2006. http://www.safcglobal.com/etc/medialib/docs/Sigma/Product_Information_Sheet/r013.Par.0001.File.tmp/r013.pdf.
[46] Nemec HW, Monig H. Investigations of radical formation and inactivation of suspended trypsin after γ-irradiation. Rad Environ Biophys 1977;14:137–45.