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Stability of new actinomycete protease subjected to UV light radiation

ABSTRACT

Two proteases (H1BT and H2BT) were obtained from actinomycete strain, isolated from Antarctic penguin excrements. In order to investigate their stability, the partially purified enzymes were subjected to UV light irradiation for up to 5 hours. Wavelength of 302 nm was used with a nominal power of 8 W. Antioxidant supplements such as dithiothreitol (DTT) and cysteine were used in order to protect the enzymes from inactivation. The protease H1BT kept more than 50% of its initial activity after 30 min of irradiation without any supplements and dropped down to 29% afterwards. The protease H2BT was stable for up to 10 min of irradiation and then lost more than 60% of its activity. Both DTT and cysteine enhanced the stability of the two proteases and thereafter they showed greater activity. In the presence of 5 mM DTT the residual activity of H1BT protease increased 1.54 times than its residual activity without supplements. For the same cysteine concentration the corresponding residual activity increased 1.18 times only. In the case of H2BT protease a similar trend was observed.

Key words: proteases, UV irradiation, enzyme stability

Introduction

Microbial proteases dominate the worldwide enzyme market, representing more than 50% of the total enzymes sales (Nejad et al., 2014). These enzymes are widely utilized in molecular biology (Mótyán et al., 2013), leather (Saran et al., 2013), food, pharmaceutical (Sumanth et al., 2006), detergents (Paul et al., 2014), textile industries (Silva et al., 2006), in peptide synthesis, in waste water treatment and biocontrol (Brandelli, 2007) and recently in poultry feed (Romero & Plumstead, 2013). Proteases are also envisaged as having extensive applications in development of ecofriendly technologies as well as in several bioremediation processes (Annamalai et al., 2013). In this regard searching for new enzymes exhibiting higher stability toward specific environmental conditions (UV light, extreme temperatures, pH values etc.) will reveal more possibilities for green process engineering.

Ultraviolet (UV) light is an electromagnetic radiation with a wavelength shorter than that of visible light. The UV wavelength range is usually divided into three sections according to the associated energy and harmful effects caused. UV-C (200-280 nm) are extremely damaging, but are absorbed by the ozone layer so do not reach the earth's surface; UV-B (280-320 nm) represents only 1.5% of the total spectrum but can penetrate to a depth of a few millimeters into the skin, causing acute chronic reactions and damage; such as skin reddening or sunburn; UV-A (320-400 nm) can penetrate into the lower layers of the skin and eye and damage DNA and intracellular macromolecules (Hollósy, 2002).

Aromatic residues in proteins structure can capture UV light and thereafter get excited and enter photochemical pathways which might have damaging effect on protein structures. However, the protein structure could be maintained stable by the present disulphide bridges which are quenchers of the excited state of the aromatic residues (Neves-Petersen et al., 2012). Reducing agents can also be used as preservatives against UV light enzyme inactivation (Lante et al., 2013).

With the ozone layer depletion we are getting more exposed to the harmful effect of UV light, and especially to UV-B rays. In this regard, naturally produced, harsh environment stable enzymes are advantageous to be used in

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green process development. Therefore, studying their stability against different ambience factors is needed.

The present investigation aimed to study the UV light stability of two proteases isolated from Antarctic penguin excrements. The protective effect of DTT and cysteine was also evaluated.

Materials and Methods

Proteases source and purity

The actinomycete strain, producer of H1BT and H2BT proteases, was isolated from Antarctic penguin excrements by Gushterova et al. (2005). The both enzymes were obtained after culture media optimization (Hristova et al., 2012). Ammonium sulphate salting out fraction (50-70% saturation) was subjected to gel filtration on Sephadex G75. Separately pooled fractions of H1BT and H2BT were further used.

Proteolytic activity

The protease activity was measured according to the method reported by Jain et al. (2012) with slight modifications. The enzyme was added to 0.6% w/v casein buffered to pH 7.0. The reaction mixture was incubated at 60°C for 10 min thereafter 0.11 M trichloroacetic acid was added to terminate the reaction. The reaction mixture was kept at room temperature for 30 min followed by centrifugation at 4500xg for 5 min. An aliquot of the supernatant was added to 0.5 M sodium carbonate solution and 1N Folin-Ciocalteu reagent and incubated for 30 min at 37°C for color development and the absorbance at 660 nm was measured. The proteolytic activity was calculated in Units per cm^3 which is defined as the amount in micromoles of tyrosine equivalents released from casein per minute and per cubic centimeter of partially purified enzyme.

UV light irradiation

The UV light irradiation was conducted according to the method described by Lante et al. (2013). Protease activity was measured at the 5th, 10th, 30th, 60th, 120th, 180th, 240th and 300th minute. The same experiment was performed in the presence of 5 mM DTT and 5 mM cysteine for both partially purified proteases H1BT and H2BT. The effect of UV irradiation on proteolytic activity was expressed as residual activity (%) as follows:

$$\%\, Residual\ activity = \frac{U.ml_t^{-1}}{U.ml_{t0}^{-1}} \times 100$$

$U.ml_t^{-1}$ – the proteolytic activity at defined time of irradiation t.

$U.ml_{to}^{-1}$ – the proteolytic activity prior to irradiation.

All results reported represent the mean of at least 3 measurements.

Results

In order to determine whether the UV light exposure affects the proteolytic activity of the two newly isolated, partially purified proteolytic enzymes, they were irradiated over the course of 300 min. It was observed that the proteolytic activity of both enzymes decreased with the irradiation progress; nevertheless they remained fully active after the 5th min of treatment (Figure 1). The protease H1BT kept more than 50 % of its initial activity after 30 min of irradiation and dropped down afterwards. In contrary, the protease H2BT was stable only for up to 10 min of UV exposure and then lost more than 60 % of its activity. However, H1BT and H2BT were inactivated after 2 h of irradiation.

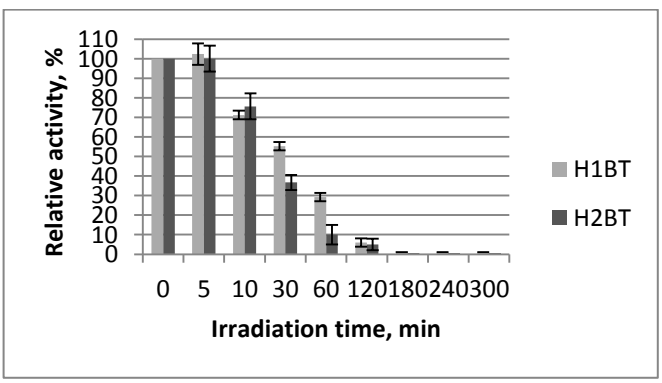

Figure 1. *Residual proteolytic activity of H1BT and H2BT proteases subjected to UV irradiation over the course of 300 min*

In order to enhance the enzyme stability and to prolong the catalytic activity of both enzymes, antioxidant reagents were used. The impact of different concentrations of DTT or cysteine on enzyme activity was also examined (not published results). 5 mM of reducing agents was found to be a suitable concentration to implement in this experiment.

Partially purified protease H1BT showed greater activity in the presence of reducing agents during the irradiation compared to the enzyme without supplements (Figure 2). The enzyme kept more than 85 % of its initial activity in the

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presence of 5 mM DTT after 30 min of exposure which is 1.56 times higher than the same time interval of the unprotected enzyme. Cysteine didn't affect the stability of protease H1BT. The enzyme showed similar or slightly higher activity during the treatment, compared to the same stage of experiment but without cysteine.

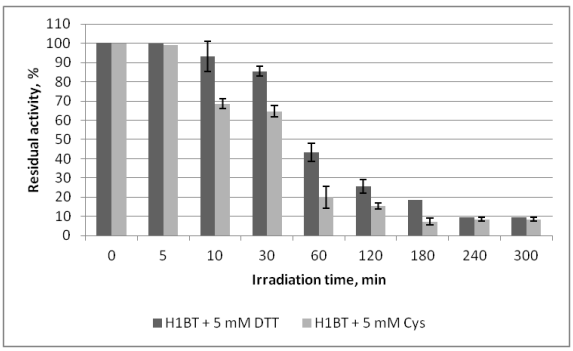

Figure 2. *Proteolytic activity of H1BT protease in the presence of 5 mM DTT or 5 mM cysteine during UV irradiation*

Figure 3 shows the residual activity of irradiated partially purified protease H2BT in the presence of 5 mM DTT or cysteine. Both reducing agents contributed considerably to the catalytic activity maintenance over the course of UV light exposure. After 60 min of irradiation the H2BT enzyme was fully inactivated. However, at the same time interval, and in the presence of DTT or cysteine, 38 % and 28 % remained proteolytic activity was observed respectively. Even after 120 min of irradiation there was still a noticeable enzyme activity.

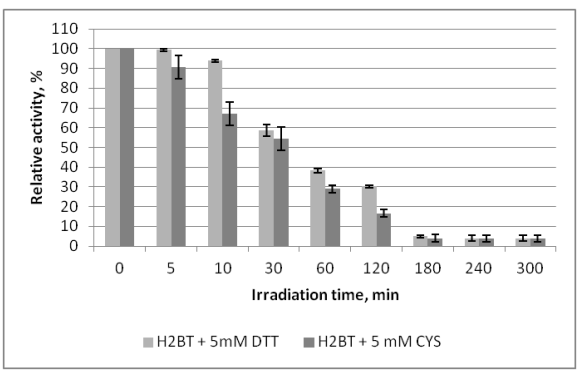

Figure 3. *Proteolytic activity of H2BT protease in the presence of 5 mM DTT or 5 mM cysteine during UV irradiation*

Discussion

UV irradiation is a technology often used in decontamination processes. The lethal effect over bacteria, viruses and parasites is attributed to many reasons (Mukhopadhyay & Ramaswamy, 2012). It is considered that the photochemical transformation of pyrimidine bases to form dimers in the DNA is one of the major lethal causes (Elmnasser et al., 2007). The impact of UV irradiation on microorganisms leads to its increased number of applications in food decontamination and food contact surfaces cleaning (Falguera et al., 2011; Mukhopadhyay & Ramaswamy, 2012). Therefore the susceptibility of food related microorganisms and enzymes on UV exposure is studied regularly in the literature.

Neves-Petersen et al. (2012) examined the impact of UV light irradiation on proteins. They concluded that during exposure of proteins the present aromatic residues got excited. Positively charged residues, the carbonyl group of the peptide chain and disulfide bridges acted as electrons scavengers afterwards. All those interactions can alter the enzyme structure. If the catalytic center is affected, then inactivation occurs. In order to protect enzyme's structure, reducing agents could be used (Lele & Russell, 2005; Lante et al., 2013). They capture the free radicals formed and protect the enzyme's structure from photooxidation.

Ibarz et al. (2009) studied the rate of inactivation of two proteolytic enzymes (carboxypeptidase A bovine pancreas solution and trypsin obtained from powdered porcine pancreas) by UV-Vis light irradiation. They demonstrated that trypsin and carboxypeptidase- A enzyme solutions were fully inactivated after 12 and 20 min of exposure, respectively. In contrast partially purified proteases H1BT kept more than 50 % of its initial activity after 30 min of UV irradiation. Other study demonstrating the harmful effect of UV light over proteolytic enzymes was conducted by Lante et al. (2013). They used commercially available enzymes and noticed that after 15 min of irradiation a significant loss of activity was observed for all the enzymes tested. Both authors reported the formation of aggregates on the course of irradiation.

The degradation of the ozone layer in Antarctica leads to higher levels of ultraviolet radiation (Takahashi et al., 2012). At the same time the microbial source of H1BT and H2BT was isolated from Antarctic penguin excrements (Gushterova et al., 2005). It is likely to presume that this could be one of

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the reasons for the higher UV light stability of the studied proteases compared to the other proteolytic enzymes studied already. The fact that partially purified enzymes were used for this experiment could also be an explanation for the observed high enzymatic stability. The presence of other proteins in the reaction mixture, that probably act as substrate, protect the catalytic center of the protease during the exposure. Even if there are aggregates formed, they didn't affect the catalytic center during the first 5 minutes of UV exposure.

In accordance with the study of Lante et al. (2013) and Lele & Russell (2005) the addition of reducing substances maintained the catalytic center active.

In conclusion the investigated proteases H1BT and H2BT showed extremely high stability when exposed to UV light irradiation. They remained fully active after 5 min of exposure and kept more than 70 % after the 10th minute. DTT or cysteine was used in 5 mM concentration in order to neutralize the free radicals formed during the UV treatment. Both antioxidants showed positive influence on H1BT and H2BT stability and prolonged their activity.

Acknowledgement

This study has been carried out with financial support from the Commission of the European Communities, FP7 Thematic Area "Aerostructures Flight physics", AAT-2010-266029 AEROMUCO. It does not necessarily reflect its views and in no way anticipates the Commission's future policy in this area.

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