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Turnover of oxidatively modified proteins: the usage of in vitro and metabolic labeling

Betul Catalgol [a,b], Tilman Grune [a,*]

[a] Institute of Biological Chemistry and Nutrition, University Hohenheim, Stuttgart 70593, Germany
[b] Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Istanbul University, Istanbul, Turkey

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ABSTRACT

Cellular reactions to oxidative stress always include a response in the protein turnover. Therefore, cellular handling of proteins is important to observe. In this method review, radioactive labeling of proteins in vitro and in intact cells is described. The use of techniques based on the radioactive quantification of amino acids is much more selective and reliable than other nonradioactive methods for studying the protein turnover of both long- and short-lived proteins. Variations of such measurements allow one to measure protein synthesis, protein degradation, formation of insoluble proteins, and, perhaps, the turnover of individual proteins.

Exposure of proteins to oxidants causes various changes in their structure. These oxidative modifications in protein structure may vary according to the kind of amino acid side chain and, in further steps, increases in surface hydrophobicity, and aggregation due to covalent cross-linking [1–4]. Susceptibility of proteins to proteolysis is increased as a result of unfolding and increased surface hydrophobicity [5,6]. Strong and perhaps chronic oxidation of proteins results in the formation of protein aggregates, known to be poor substrates for proteolysis, and may inhibit proteolysis [7]. Therefore the determination of protein aggregation and proteolytic degradation in cells provides valuable information with regard to oxidative modifications.

Early work done by Schimke et al. [8–12] used radioactively labeled or nonproteogenic amino acids. However, the most convenient way of measuring proteolysis of modified proteins in functionally intact cells is accepted to be the use of techniques based on the radioactive quantification of amino acids using liquid scintillation counting [13]. This method has been used in many studies to measure the changes in protein degradation [6,14–16] and protein aggregate formation [17,18] after various treatments.

Liquid scintillation counting is an analytical technique for measuring radiation from β-emitting nuclides such as ^{14}C, ^{3}H, ^{35}S, ^{32}P, and ^{63}Ni and is the most frequently used method to detect radioactivity in biologic samples labeled with isotopes [19,20]. Samples are dissolved or suspended in a cocktail containing an aromatic solvent and small amounts of other additives known as fluors. Sample preparation is critical for proper counting because the energy transfer process necessitates close contact between the sample and the fluor in the counting solution. β particles emitted from the sample transfer energy to the solvent molecules, which in turn transfer their energy to the fluors; the excited fluor molecules dissipate the energy by emitting light. In this way, each β emission results in a pulse of light. The primary and most extensively used scintillation cocktails contain 2,5-diphenyloxazole, toluene, xylene, and dioxane. During measurement, the samples are placed in small transparent or translucent (often glass or plastic) vials that are loaded into an instrument known as a liquid scintillation counter. The counter has two photomultiplier tubes connected in a coincidence circuit. The coincidence circuit ensures that genuine light pulses, which reach both photomultiplier tubes, are counted, whereas spurious pulses (due to noise, for example), which would affect only one of the tubes, are ignored [21].

In this review, we describe the proteolytic degradation and protein aggregate formation determinations using liquid scintillation counting under oxidative stress conditions.

Principles

Proteolysis determinations include either the degradation of metabolically radiolabeled cellular proteins or the degradation of radiolabeled "foreign" proteins or peptides during incubation with (unlabeled) cell lysates [15]. Additionally, with the metabolic labeling of intracellular proteins additional information can also be obtained, including the formation of insoluble protein complexes due to

* Corresponding author. Fax: +49 711 459 23386.
E-mail address: grune@uni-hohenheim.de (T. Grune).

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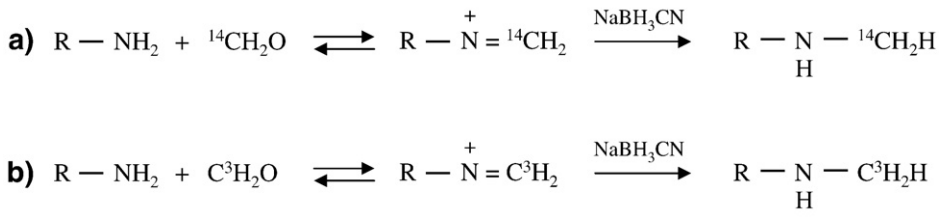

Scheme 1. Conversion of protein amino groups to mono- and dimethylamino groups, the sites of label incorporation. (a) Using ^{14}C and (b) using ^{3}H.

aggregation of unfolded, denatured proteins [18]; the turnover of single proteins; and more.

Radioactive labeling of proteins in vitro

^{125}I, ^{3}H, or [^{14}C]-containing groups are attached to the proteins. However, because ^{125}I irradiates high-energy irradiation, the use of this isotope is not recommended for testing the degradation of oxidized proteins [22,23]. Hence ^{3}H or ^{14}C labeling is used more often. Radioactively labeled (^{3}H or ^{14}C) formaldehyde is used and added to amino groups by reductive alkylation (Scheme 1). Sodium borohydride or sodium cyanoborohydride is used as reducing agent for Schiff bases [24,25].

Quantification of radiolabeled foreign protein degradation

The labeled proteins are incubated afterward with the cell lysate, protease mixture, etc., for a given amount of time under optimal conditions for the proteases. After incubation of the labeled protein with this biological sample, degradation is determined using liquid scintillation counting after trichloroacetic acid (TCA) precipitation [15,16]. If protein concentrations are very low, bovine serum albumin (BSA) has to be added as a precipitation carrier.

It is important to note that such methods reflect more the maximal capacity of a proteolytic system than the real capacity of this system in a living cell. Therefore, the degraded part of the protein substrate (% degradation) has to be small (not above 20%) to ensure the V_{max} of the proteolytic degradation of the substrate.

(*Note:* This is even more important in the case of oxidized proteins. In such a case it is not known how efficient the oxidation procedure is, how different the oxidation products are, or what their proportion of the total radioactivity is.)

Metabolic labeling of cellular proteins with amino acids

The most important step is the labeling of proteins in intact cells by incorporation of exogenously added radioactive amino acids. Metabolic labeling of cellular proteins is achieved by placing cells in a nutritional medium containing all components necessary for the growth of the cells in culture, except for one amino acid, which is substituted by its radiolabeled form. The radiolabeled amino acids are transported across the plasma membrane by carrier-mediated systems and, once in the cytosol, are loaded onto tRNA molecules before being incorporated into newly synthesized proteins [26].

Because metabolic labeling techniques use the metabolic machinery of the cell to incorporate radiolabeled amino acids, there are limitations on the type of radiolabeled amino acids that can be employed. The list of potential precursors is restricted to L-amino acids normally found in proteins, in which one or more atoms are substituted by a radioisotope [26] (Table 1).

Cells are able to synthesize nonessential amino acids from other compounds, and using nonessential amino acids in radiolabeling will cause a reduction in the specific activity by dilution with the endogenously synthesized amino acids. Therefore, essential amino acids should be favored for metabolic labeling [26]. Sulfuric amino acids, such as methionine and cysteine, are conveniently labeled with ^{35}S [27]. [^{35}S]Methionine/cysteine is the most frequently used radiolabeled amino acid because of its high specific activity (>800 Ci/mmol). For proteins that contain little or no methionine/cysteine, other amino acids labeled with ^{3}H and ^{14}C can also be obtained [22]. [^{3}H]Leucine, with activity of up to 190 Ci/mmol, is a good alternative to ^{35}S-labeled amino acids. But several problems can arise when using certain ^{3}H-labeled amino acids owing to their participation in metabolic pathways. When choosing the labeled amino acids, specific activities and half-lives have to be considered.

In general, the duration of labeling should be dictated by the half-life of the protein or protein fraction of interest. Short-time labeling of up to 2 h will result in radioactivity incorporation in "short-lived" proteins or proteins that are synthesized in large quantities. Longer incorporation times will lead to the detection of "long-lived" proteins. This protein fraction can be further defined if a 16-h labeling "pulse" is, for example, followed by a "cold chase" of 2 h (for degradation of short-lived proteins) before the start of the actual experiment [13].

In the case of cell lines it can be important to know the cell cycle duration of the cell line used. For example, a cell type that doubles

Table 1
Radiolabeled amino acids used in metabolic labeling of proteins [26]

Amino acid[a]	Freq (%)[b]	Radio-isotope	Sp act (Ci/mmol)	Organ or tissue[c]	Cells[c]
Leucine	10.4	^{3}H	5–190	Brain, heart	Ovary cells, neurons, pneumocytes
		^{14}C	0.22–0.3	Liver, muscle	Fat cells
Lysine	7.0	^{3}H	40–110	Kidney, brain, retina, thymus	Ovary cells
		^{14}C	0.22–0.26	Kidney, brain, liver, spleen	Nerve cells, blood cells
Valine	6.2	^{3}H	10–65	Liver, brain, heart, jejunum	Astroglial cells, hepatoma cells
		^{14}C	0.18–0.22	Liver, brain, kidney, skeletal muscle	Blastocysts
Threonine	5.6	^{3}H	5–25	Bronchial submucosal gland, colonic mucosa	
Histidine	2.5	^{3}H	30–70	Brain, intestine, nerve tissue	
Isoleucine	2.9	^{3}H	30–140	Brain, liver	Erythrocytes
		^{14}C	0.22–0.26	Brain, liver, white muscle	Fibroblasts
Cysteine	3.4	^{35}S	>800	Liver, brain, pancreas, kidney, heart	Leukocytes, fibroblasts, K562 cells, epithelial cells, etc.
Methionine	1.8	^{35}S	>800	Brain, epididymis	Lymphoblasts, reticulocytes, chromaffin cells, fibroblasts, ganglion cells, etc.

[a] All amino acids are in the L configuration.
[b] Frequency of amino acid residues in proteins.
[c] Organs, tissues, and cells that were used for radiolabeling with the corresponding amino acid.

every 24 h will also produce a whole proteome set within this time. For numerous cellular proteins involved in the cell cycle, the lifetime of a single protein depends on the cell cycle. On the other hand, long-lived proteins, once labeled, might exist over several cell cycles and be transferred after the labeling to the daughter cells, functioning there also. Because a new set of these proteins will be produced, the specific radioactivity of the protein will decrease; in other words, the label is diluted. If quantified as label per milligram protein, this will look as if the protein has a shorter half-life. Because it is difficult to account for all these theoretical considerations, it is of utmost importance to perform the adequate control measurements.

Quantification of protein synthesis

The incorporation of radioactivity into the intracellular protein pool can be used as a measure of protein synthesis. Here it is recommended that rather short labeling times be used because otherwise multiple factors might influence the measurement. The incorporated radioactivity is determined as either the difference between the added radioactivity and the removed radioactivity from the tissue culture dish or the amount of resolved radioactivity after TCA precipitation of proteins. Both methods may have problems based on the precipitation of small amounts and difficulties in a 100% controlled solution of the precipitate or on the potentially large errors in volume measurements and calculations, and care should be taken to manipulate the cell number used or the time of incorporation to ensure that a sufficient amount of radioactivity is incorporated.

Quantification of proteolysis in intact cells after labeling

To determine the proteolysis rate the cell labeling medium has to be removed and the cells have to be washed. Afterward normal tissue culture medium (containing a high concentration of the amino acids used for labeling, but in a nonradioactive form) has to be added. This is required to ensure that after a protein is degraded, and the radioactive amino acid is liberated, it is not used for protein synthesis again. For the measurement of proteolysis two methods are possible. One is based on the assumption that the exchange of amino acids between the cytosol and the extracellular medium is very quick (at least much faster than proteolysis processes, which often take hours). If this is true, the measurement of TCA-soluble radioactivity in the medium of the tissue culture is sufficient. (*Note:* This assumption has to be tested for each cell line used and for each amino acid used for labeling.) If this assumption is not true (or for testing whether it can be assumed), proteolytic degradation of metabolically labeled cellular proteins should be quantified either by precipitating the cellular proteins, resolving them, and counting the liquid scintillation or by using the whole content (medium plus cells) of the tissue culture well, precipitating it with TCA, and measuring the released radioactivity. To do so, proteins will be precipitated in ice-cold TCA with a final concentration of 10% and centrifuged at 14,000 *g* for 10 min [16], followed by scintillation counting of supernatant. Performing such a procedure allows counting of all amino acids and peptides with a molecular weight below 5 kDa.

Quantification of protein aggregates in intact cells after labeling

The solubility of proteins in a detergent cocktail is used as a quantitative indicator of protein aggregation [18]. After metabolic labeling, cells are often treated with oxidative stress, and the formation of protein aggregates is the readout. Proteins, which are released by sonication of the cell pellets, are solubilized in a detergent cocktail. Detergent-soluble proteins are counted to determine the decrease in the solubility and to quantify the protein aggregates; detergent-insoluble proteins are counted after solubilization in NaOH [17].

Possible extensions of metabolic radiolabeling

The methods described here are only a basic set of methods using scintillation counting in oxidative stress related protein turnover research. Of course the effect of oxidative stress depends on the time schedule of the given experiment. If the cell is first stressed and afterward the radiolabeling is performed, the influence of oxidation on the protein synthesis is tested. On the other hand, applying the oxidative stress after the radiolabeling will result in a measurement of the degradation of the radiolabeled, oxidized proteins. Once cells are harvested and lysed, such cell lysates can be used for further analysis to determine the turnover of specific proteins. This can be done by electrophoresis [28] or immunoprecipitation followed by electrophoresis and counting [29,30]. Furthermore, in vitro-labeled protein can also be used to test the degradation of extracellular proteins in tissue culture [31], or even the uptake of parts of cells or whole cells can be followed [32]. In some cases it is even possible to label not the protein, but the modification itself, as in the use of radioactive glucose to form AGE (advanced glycation endproducts) derivatives of proteins [33].

Therefore, the possibilities of using metabolic or in vitro labeling of proteins are many, and variations in the routine methods often lead to new and interesting results.

Materials

Reagents

The following is a list of the reagents for labelling of proteins and turnover measurements (use the highest purity available):

Tris
Tris–HCl
Potassium chloride (KCl)
Magnesium acetate tetrahydrate ($Mg(CH_3COO)_2{\cdot}4H_2O$)
Magnesium chloride hexahydrate ($MgCl_2{\cdot}6H_2O$)
1,4-Dithiothreitol (DTT)
D-(+)-Sucrose
2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (Hepes)
Ethylenediaminetetraacetic acid disodium salt dihydrate ($EDTA–Na_2{\cdot}2H_2O$)
Sodium chloride (NaCl)
EDTA anhydrous
Glycerol
Octylphenolpolyethylenglycol ether (Nonidet P-40, NP-40)
[^{14}C]Formaldehyde
[^{35}S]Methionine
Sodium cyanoborohydride ($NaBH_3CN$)
Sodium deoxycholate
Sodium dodecyl sulfate (SDS)
Triton X-100
Dulbecco's modified Eagle's medium without L-methionine or L-cystine
Dulbecco's modified Eagle's medium
TCA
Albumin bovine fraction V (BSA)
Dulbecco's PBS
Sodium hydroxide (NaOH)
Scintillation cocktail (e.g., Ultima Gold; Perkin–Elmer 6013326)

Equipment

The following is a list of the equipment used:

Laminar flow bench for cell culture steps

Tissue culture incubator
Sterile 5-ml pipettes
Sterile 10-ml pipettes
Sterile Pasteur pipettes
Pipettes (0.5–10, 2–20, 20–200, 200–1000 μl)
Sterile cell scraper
Sterile 0.5-ml Eppendorf tubes
Liquid nitrogen box
Water bath
Dry ice
Dialysis apparatus
Centrifuge (refrigerated)
Vortexer
Sonicator
Liquid scintillation counter
Liquid scintillation Pico Prias counting vials

Reagent Setup

The proteolysis buffer contains 0.45 M Tris, 90 mM KCl, 15 mM $Mg(CH_3COO)_2{\cdot}4H_2O$, 15 mM $MgCl_2{\cdot}6H_2O$, pH 8.2, 1 mM DTT and is stored at 4°C. Add the DTT just before use. This buffer is optimal when the proteasomal system is expected to degrade the proteins [7,13].

The lysis buffer for the cytosol is made up of 0.25 M D-(+)-sucrose, 25 mM Hepes, 10 mM $MgCl_2{\cdot}6H_2O$, 1 mM EDTA–Na_2, pH 7.8, 1 mM DTT and is stored at 4°C. Again, add the DTT just before use.

To obtain 1 M DTT, dissolve 0.5 g DTT in 3.2 ml ice-cold double-distilled H_2O.

The lysis buffer for the nucleus contains 10 mM Hepes, pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA.

The extraction buffer for the nucleus contains 50 mM Hepes, pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol.

To make 20% TCA, dissolve 20 g TCA in 100 ml double-distilled H_2O.

To make 10% BSA, dissolve 0.2 g BSA in 2 ml double-distilled H_2O.

The detergent cocktail is made up of 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS in 10 mM Tris–HCl, 1 mM EDTA, pH 8.

To obtain 1 N NaOH, dissolve 2 g NaOH in 50 ml double-distilled H_2O.

Procedure

(1) Degradation of radiolabeled foreign proteins during incubation with unlabeled cell lysates

Note: Each procedure described must be optimized for each cell type, labeled amino acid, and protein under study.

(A) Lysis of the cells for proteolytic activity in the cytosol

(i) Wash approximately 1×10^6 cells twice with PBS after oxidative stress (control and treated groups).
(ii) Pipette 300 μl cold lysis solution containing 1 mM DTT onto the cells (the amount can be arranged according to the cell type).
(iii) Scrape the cells with the cell scraper and transfer them into an Eppendorf tube.
(iv) Destroy the cell membranes with three freeze–thaw cycles in liquid nitrogen and a 40°C water bath.
(v) Remove nonlysed cells, membranes, and nuclei by centrifugation at 14,000 *g* for 30 min at 4°C.
(vi) Use the supernatants for the cytosolic proteolysis assay.

- **Critical step:** All steps must be done on ice to prevent changes in enzymatic activity.

(B) Lysis of the cells and nuclear extraction for proteolytic activity in the nucleus

(i) Wash 1×10^6 cells once with ice-cold PBS (control and treated groups).
(ii) Scrape cells with ice-cold PBS into an Eppendorf tube.
(iii) Centrifuge at 2000 rpm (higher rpm if there is no pellet) for 5 min at 4°C.
(iv) Remove the supernatant and add lysis buffer with the final volume equivalent to five times the pellet volume (50 μl for 10 μl pellet) and incubate on ice for 10 min.
(v) Add 10% NP-40 in the amount necessary to obtain a final concentration of 1%, mix by pipetting, and centrifuge at 15,000 rpm for 1 min at 4°C.
(vi) Remove and discard the supernatants. Add the same volume of extraction buffer as lysis buffer and incubate on ice for 20 min with vigorous vortexing every 5 min.
(vii) Centrifuge at 15,000 rpm for 10 min at 4°C and use the supernatants for the assay.

- **Critical step:** All steps must be done on ice to prevent changes in enzymatic activity.

(C) Labeling of the foreign proteins

(i) Prepare protein solution at a concentration>2 mg/ml and dialyze against double-distilled water.

Note: Many commercially available protein preparations contain substances such as EDTA, salicylate, or ammonium sulfate for preservation. All of these additives have significant effects on radical/oxidant reactions, and most of them also affect proteases and proteolysis. To remove these contaminants, extensive dialysis is required before use.

(ii) To label proteins by reductive methylation using [^{3}H] or [^{14}C] formaldehyde and sodium cyanoborohydride, put 50 mg of dialyzed proteins in 19 ml sodium borate on ice in a flask, add 5 mCi formaldehyde, and let it react for 5 min. Add 500 μl of 5 mg/ml sodium cyanoborohydride slowly over 1 h to the labeled protein.

- **Critical step:** Avoid a heavy reaction with heating. Please note that some radioactivity might evaporate—take caution.

(iii) Extensively dialyze the samples against PBS overnight at 4°C. Such samples can be stored frozen (−80°C) for long periods (sometimes years).
(iv) Oxidize radiolabeled protein using different vehicles. This has to be done the day before the degradation experiment. Dialyze again overnight. Oxidized proteins cannot be frozen without changing their structure.

- **Caution:** When working with radioactive materials, take appropriate precautions to avoid contamination of the surroundings. Perform the experimental steps and dispose of the waste in a properly designated area.

(D) Incubation of radiolabeled foreign proteins with unlabeled cell lysates and measurement of degradation

(i) Add 10–20 μCi labeled proteins to cytosol or nuclear lysates of 5×10^6 unlabeled cells in the proteolysis buffer for measurement of proteolysis over a time period.
(ii) Add an equal volume of 20% TCA to the cell supernatant and incubate for 2 h at 4°C. Sometimes (protein concentration below 1 mg/ml) it is better to use 10% (w/v) BSA as a carrier for optimal precipitation.
(iii) Centrifuge at 14,000 *g* for 10 min at 4°C.
(iv) Count the radioactivity in the supernatant in the liquid scintillation counter.
(v) Calculate the percentage of degradation after TCA precipitation as (acid-soluble counts − background counts)/(total counts − background counts)×100.

For this calculation, the background counts are from vials with scintillation cocktail only, whereas for total count measurements water is added instead of TCA.

- **Critical step:** Proteins should be assessed for oxidation-induced proteolytic susceptibility immediately after exposure to oxidants. Freezing also affects the stability of oxidized proteins and may therefore modulate the effects of oxidation.

(2) Measurement of proteolytic degradation and aggregate formation of cellular proteins

(A) Metabolic labeling of cellular proteins for proteolysis and aggregation measurements

(i) Wash 1×10^6 cells three times with PBS.
(ii) Incubate in methionine- and cysteine-free tissue culture medium spiked with 0.5 mCi [^{35}S]methionine/cysteine at 37°C for 16 h (long-lived proteins) or other time period as appropriate.
(iii) Wash the cells three times with PBS and collect all of the washout for subsequent estimation of label incorporation.
(iv) Add appropriate growth medium supplemented with 10 mM methionine/cysteine and incubate cells for some time (2 h) in the incubator for the turnover of the bulk of the short-lived proteins (cold chase). This step is not always required.
(v) Treat the cells with the desired oxidant.
(vi) Wash the cells twice again with PBS and collect all of the washout for subsequent estimation of label incorporation.
(vii) Add appropriate growth medium supplemented with 10 mM methionine/cysteine and incubate for desired time.

(B) Measurement of degradation of metabolically radiolabeled cellular proteins

(i) Add an equal volume of ice-cold TCA (20%) to the media that are collected from the incubation periods at the desired time points.
(ii) Mix growth medium (unlabeled) with an equal volume of ice-cold TCA (20%) to estimate background radioactivity.
(iii) Keep all samples on ice for 30 min and centrifuge at 14,000 *g* for 10 min at 4°C.
(iv) Transfer aliquots from the supernatants of samples and background carefully, dilute about 100-fold with scintillation cocktail, and count the acid-soluble radioactivity.
(v) Count the aliquots of the radioactively spiked tissue culture medium and the washout as well.
(vi) Calculate the radioactivity incorporated into the cellular proteins as the difference between the amount of label used for the pulse and the content of label in the washout. Subtract the background from the sample counts and do the calculation as follows:

Percentage degradation
= (acid−soluble sample counts/incorporated counts)×100

Incorporated counts
= counts in the radioactivity medium added to cells
$-\sum$(washouts)

- **Critical step:** To calculate the incorporated radioactivity it is crucial to measure all volumes exactly better than normally done in tissue culture procedures. In particular, washing steps with low radioactive counts might have larger volumes and, therefore, dramatically influence the result.

(C) Measurement of aggregation of metabolically radiolabeled cellular proteins

(i) Scrape the cells into cold PBS and prepare cell extracts by sonication of cell pellets.

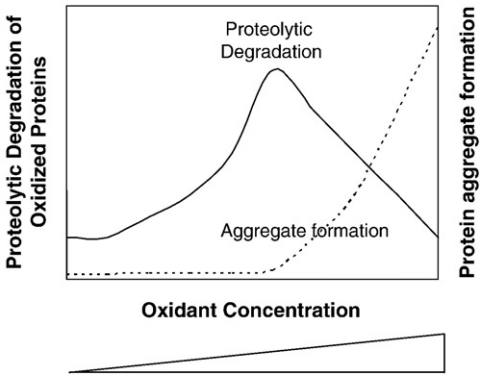

Fig. 1. Diagram describing the oxidant concentration dependence of the proteolytic susceptibility of oxidized proteins (proteolytic degradation) and the aggregate formation with severe oxidation. Such aggregates are poor proteolytic substrates and in turn might inhibit proteases and, therefore, proteolytic degradation.

(ii) Take 100 µl of the extracts for total cell protein counting.
(iii) Lyse (or resuspend the cell pellets) at 4°C for 15 min in detergent cocktail.
(iv) Centrifuge the cell lysate at 13,000 *g* for 10 min at 4°C. The supernatant contains detergent-soluble proteins, whereas the pellet contains detergent-insoluble proteins.
(v) Wash the detergent-insoluble pellet three times with PBS and dissolve in 1 N NaOH.
(vi) Transfer aliquots from the supernatants of the detergent-soluble and insoluble samples, dilute about 100-fold with scintillation cocktail, and count the radioactivity.
(vii) Count aliquots of the washout as well.
(viii) Subtract the background from the sample counts and do the calculation as follows:

Percentage of detergent–insoluble/soluble proteins
= (detergent–insoluble/soluble protein count
÷ incorporated count)×100

Expected results

In the case of long-term labeling with amino acids, the incorporation is expected to be 30 to 60%. Shorter labeling procedures are very often less efficient. During the counting process, it is always better to have a high counting efficiency. These conditions affect the efficiency and reliability of the results.

After oxidative stress in cells, it is also important to determine the time points at which proteolysis and aggregates are measured.

In the proteolysis results, mild oxidation generally increases the proteolysis, and with an increase in oxidation proteolysis is known to be inhibited. Our explanation of these findings is that mildly oxidatively damaged proteins are susceptible to degradation, whereas extremely damaged proteins form cross-links and aggregates that are poor substrates for proteolysis (Fig. 1) [34]. Therefore, it is always required to use several concentrations of the oxidant to test the influence on the proteolytic susceptibility.

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