An electrophoretic approach to screen for glutamine deamidation

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Protein deamidation is a posttranslational modification with important implications in physiology and medicine. There is, however, no simple technique for a rapid screening of protein deamidation. The deamidating activity of transglutaminase was applied to establish a simple method for the screen of protein deamidation using recombinant human growth hormone, a rat hippocampal membrane fraction, and a cell homogenate enriched in 5-hydroxytryptamine-1A receptor as model systems. Here we report a simple, economic, and fast approach to assess protein deamidation by two electrophoretic methods: differential cleavage on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) via in situ V8 protease digestion and the principle of spot shifting via blue native (BN)–PAGE/two-dimensional (2D)–SDS–PAGE/immunoblotting.

Many proteins are susceptible, both in vivo and in vitro, to deamidation that can lead to alterations in their structure and biological functions [1]. The physiological relevance of protein deamidation can be inferred from the essential roles fulfilled by this posttranslational modification (PTM): labeling of proteins for degradation (N-end rule) [2], protein aggregation with repercussions in important neurodegenerative diseases [3], and targeting of proteins to specific subcellular locations [4]. Virulence and cytotoxic mechanisms induced by bacterial pathogens in eukaryotic cells [5], and being involved in the aging process as well as in the molecular basis of health disorders such as arthritis and celiac disease [6]. Despite its important biological function, there is limited information on experimental methods for economic and rapid determination or screen of protein deamidation [7,8].

Deamidation is a well-known artifact generated by analytical steps, but technical processing of protein drugs may seriously affect protein function by deamidation, probably leading to changes of chemical, biological, and pharmacological properties [9]. Of note, the finding that receptors can be rapidly regulated reciprocally by deamidation has fueled major interest in this process in protein chemistry and biology [10]. Deamidation of asparagine residues occurs both in vitro and in vivo, generating aspartic acid and isoaspartic acid. Glutamine deamidation takes place by hydrolysis under acidic conditions and proceeds via a glutarimide intermediate at neutral or alkaline conditions [1,11]. In general, nonenzymatic glutamine deamidation has been shown to be slower than asparagine deamidation.

Tissue transglutaminases (TGs, EC 2.3.2.13) are calcium-dependent enzymes that catalyze transamidation and PTMs of proteins [12]. Their main activity is cross-linking of proteins involving a glutamine residue in a substrate and a lysine residue in a cosubstrate. Besides cross-linking activity, in the absence of cosubstrate, TGs also catalyze deamidation of glutaminyl residues in peptides and proteins. Many amines and polyamines are substrates of TGs with important consequences for the modified proteins. Recently published reports indicate the significance of protein deamidation by TGs [13,14]. A precise consensus sequence for the TG is not well known, and the ratio between deamidated and transamidated reaction products is highly substrate dependent [15].

Gel electrophoresis is a very powerful tool for the separation and characterization of polypeptide chains and proteins in complex biological samples. Here an electrophoretic method was modified and used to assess glutamine deamidation from the principle used for peptide mapping [16]; enzymatic or spontaneous protein deamidation can be assessed in this analysis. Furthermore, a two-dimensional method was optimized by combination of blue native–polyacrylamide gel electrophoresis (BN–PAGE) and sodium dodecyl sulfate (SDS)–PAGE; in this way, we are able to detect deamidation both in native protein complexes and in denatured states.
Recombinant human growth hormone (rhGH, Nutropin, Genentech, South San Francisco, CA, USA) was treated with TGS from guinea pig liver (Sigma, product no. T5398) in TG buffer (50 mM Tris–HCl, 150 mM NaCl, and 2 mM CaCl₂, pH 7.5) at different enzyme amounts and separated on SDS–PAGE and two-dimensional gel electrophoresis (2DE). No differences in electrophoretic mobility were observed on SDS–PAGE (see Supplementary Fig. 1a in supplementary material), but on 2DE several acidic spots for rhGH were detected (Supplementary Fig. 2). TG-treated rhGH samples were briefly (~10 min) stained in a solution containing 0.1% Coomassie blue, 50% methanol, and 10% acetic acid and then briefly destained in a solution of 5% methanol and 10% acetic acid. Subsequently, each band was cut out with a razor blade, trimmed to 5 mm width, and incubated for 30 min with V8 buffer (50 mM sodium phosphate, 0.1% SDS, and 1 mM ethylenediaminetetraacetic acid [EDTA], pH 7.2). Sample wells of second gradient SDS–PAGE gel (7.5–20%) were filled with V8 buffer, and each gel slice was washed to the bottom of a well. Spaces around slices were filled by overlaying each slice with V8 buffer containing 10% glycerol, and then a given amount of V8 enzyme (Endoproteinase Glu-C from Staphylococcus aureus V8, Roche, product no. 10791156001) was loaded to each well. Electrophoresis was stopped after 1 h for in situ V8 digestion when the bromophenol blue dye approached the bottom of the stacking gel. The length of the stacking gel was longer than normal SDS–PAGE (i.e., ~5 cm). After proteolysis, samples were rerun to separate cleaved peptides on gradient SDS–PAGE (7.5–20%). The V8–PAGE gel was stained by Coomassie blue, and deamidation of rhGH was revealed by different mobility of rhGH peptides generated by V8 (Fig. 1).

Rat hippocampi and tsA201 cells overexpressing rat 5-hydroxytryptamine-1A receptor (5HT1A-R) were homogenized, and samples were incubated for 1 h with TG or TG buffer alone and subsequently loaded onto BN–PAGE gels. The BN–PAGE gel buffer contained 500 mM 6-aminocaproic acid and 50 mM Bis–Tris (pH 7.0); the cathode buffer consisted of 50 mM Tricine, 15 mM Bis–Tris, and 0.05% (w/v) Coomassie G–250 (pH 7.0); and the anode buffer was 50 mM Bis–Tris (pH 7.0). The voltage was set to 50 V for 1 h and 75 V for 6 h, and it was increased sequentially to 400 V (maximum current = 15 mA/gel, maximum voltage = 500 V) until the dye front reached the bottom of the gel [17] (Fig. 2). For the detection of 5HT1A-R in the individual native complexes, lanes from BN–PAGE were cut and equilibrated for 1 h in an equilibration buffer (1% [w/v] SDS and 1% 2-mercaptoethanol) with gentle agitation and then briefly rinsed with Milli-Q water. Gels were performed in a PROTEAN II xi Cell using 5% stacking and a 5 to 18% separating gel (Supplementary Fig. 1b). The 5HT1A-R was revealed by Western blotting using specific rabbit polyclonal antibody (GenScript, Piscataway, NJ, USA). As shown in Fig. 2, there was a clear electrophoretic mobility up-shift of 5HT1A-R on BN/SDS gel electrophoresis following TG treatment. A corresponding electrophoretic shift in mobility was also observed when the rat hippocampal membrane fraction was treated with TGS, analyzed by BN–PAGE/2D SDS–PAGE gels, and revealed with 5HT1A-R antibodies by Western blotting (Fig. 2).

In the BN–PAGE system, TG-treated proteins migrate faster than untreated samples because deamidation introduces more negative charges in the protein [5]. In our case, TG treatment using BN–PAGE combined with immunoblotting was not suitable to show electrophoretic shifts because the signal for the 5HT1A-R on immunoblotting was weak, probably because of reduced immunoreactivity [18], and it was hard to clearly differentiate between deamidated and nondeamidated bands due to their low resolution in BN–PAGE/immunoblotting (Supplementary Fig. 3). The possibility that cross-linking by TG treatment would have occurred under our experimental conditions is highly unlikely because protein cross-linking occurred only after 8 h of incubation with TGS and not at 1 h (Supplementary Fig. 4). Therefore, SDS–PAGE following separation on BN gels was carried out and indeed showed electrophoretic shifts of the receptors from tsA201 cells with 5HT1A-R enrichment and from the rat hippocampal membrane fraction. As compared with untreated receptor protein, spots representing the TG-treated 5HT1A-R were up-shifted; increased negative charges of deamidated proteins do interfere with binding of SDS with the proteins, which may weaken the denaturation effects of SDS to form the compact rod complex for migrating effectively. In addition, carboxyl groups increased by deamidation may intensify electrostatic repulsion within molecules, thereby producing molecules with expanded shape for which it is difficult to migrate through the gel matrix [19].

To show that deamidation following TG treatment did occur, mass spectrometric determinations of deamidation on four 5HT1A-R peptides were carried out on protein spots using tandem nano liquid chromatography–tandem mass spectrometry (nanoLC–MS/MS) (ion trap, HCT, Bruker, Germany) as previously [20]. As shown in Supplementary Fig. 5 (showing mass spectra of pairs with and without TG treatment), the four peptides tested were revealing glutamine deamidations following TG treatment (one per peptide) on Q67, Q257, and Q408.

The Cleveland method of in situ V8 proteolytic digestion combined with the BN–2DE–SDS–PAGE/Western blotting system may be a useful screening approach to propose deamidation of both asparagine and glutamine residues in pure protein formulations and crude protein extracts. V8 protease can also cut newly formed aspartic acid residues after asparagine deamidation. This experimental setup is based on orthogonal methods; one is based on the appearance of novel peptide bands after proteolysis, whereas the other exploits differences in electrophoretic mobilities in deamidated versus nondeamidated proteins. Obviously, when using complex protein mixtures, deamidation should be verified in a subsequent step by mass spectrometry [21].

![Fig.1. In situ V8 digestion of rhGH in SDS–PAGE. The SDS–PAGE gel was stained by a colloidal blue staining kit. Lane 1: control (untreated rhGH); lane 2: rhGH with TG buffer; lane 3: rhGH with 0.125 U/ml TG; lane 4: rhGH with 0.25 U/ml TG. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)(Image 52x89 to 265x358)
Fig. 2. Electrophoretic mobility shift of in vitro TG-treated 5HT1A-R overexpressed tsA201 cell homogenates and a rat hippocampal membrane fraction (HMF). tsA201 cells overexpressing 5HT1A-R-YFP (yellow fluorescent protein) (A) and rat hippocampi (B) were homogenized, and samples were incubated for 1 h with TG or TG buffer alone and subsequently loaded onto BN–PAGE gels. The spot shift of deamidated 5HT1A-R was revealed by Western blotting using a specific rabbit polyclonal antibody. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jab.2012.05.016.

References