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A Highly Sensitive Method for the Determination of Protein Bound 3,4-Dihydroxyphenylalanine as a Marker for Post-Translational Protein Hydroxylation in Human Tissues *ex vivo*

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A highly sensitive, specific and tissue-independent method is described to evaluate oxidative stress-mediated protein hydroxylation in red blood cells, frontal cortex, and liver by HPLC separation and electrochemical detection of protein-bound 3,4-dihydroxyphenylalanine (DOPA) following gas-phase amino acid hydrolysis of tissue protein extracts containing exclusively proteins larger than 3 kDa. Simultaneous measurement of protein tyrosine (Tyr) content using fluorescence detection results in a tissue specific DOPA/Tyr ratio that may reflect oxidative stress-mediated protein modifications in disease, or following the exposure to oxidative stress-inducing agents.

Keywords: Oxidative stress, protein hydroxylation, method, human tissue, red blood cells, protein-bound DOPA

INTRODUCTION

Oxidative stress has been implicated as an initiating or at least aggravating factor in neurodegenerative diseases, atherosclerosis, stroke, and inflammatory diseases. Since reactive oxygen species (ROS) are short-lived reactive species, the involvement of ROS in biological tissues is determined by the evaluation of ROS-modified marker molecules. In principle, ROS react unspecifically with nearly any kind of biomolecule depending on the site of their production. Protein-bound 3,4-dihydroxyphenylalanine (PB-DOPA[1]) is suggested to be a relatively stable marker of oxidative stress. PB-DOPA is reported to be a product of tyrosine (Tyr) oxidation by mushroom tyrosinase[1] and has been found in atherosclerotic plaques in humans[2] and in human apolipoprotein B.[3] Currently it is believed that PB-DOPA may be generated by hydroxyl radical attack on protein Tyr, since the involvement of a putative human tyrosinase

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or other Tyr hydroxylating enzymes using proteins as substrates has not been clearly demonstrated. Hydroxyl radicals can arise from the Fenton-type reactions of hydrogen peroxide with Fe^{2+} or Cu^{+} (for a review see Ref. [4]). Since Tyr-hydroxyl binds to transition metals, it is likely that especially in enzymes that produce hydrogen peroxide and contain Tyr at the vicinity of the catalytic site (such as monoamine oxidases) oxidation of protein-bound Tyr may occur. It is thus believed that in proteins, DOPA is a relatively stable indicator of radical attack since its half-life might be only determined by the half-life of the protein itself, and further redox reactions induced by strong oxidizing species such as Fe^{3+} or Cu^{2+} which would result in quinone species formation.[1] In this respect, PB-DOPA has been demonstrated to promote further radical-generating events subsequently inducing damage to other biomolecules such as DNA.[5]

Current fluorimetric assays for the detection of PB-DOPA[6] were shown to be less sensitive than the electrochemical detection of DOPA. The former studies mainly used model proteins to investigate the biochemistry of *in vitro* oxidation of Tyr induced by ionizing irradiation and the iron/EDTA/ascorbate system in proteins.[1,7] With electrochemical detection the sensitivity of the determination of basal levels of PB-DOPA and other Tyr congeners such as 3-NO_2-Tyr in various tissues from healthy individuals and under pathological conditions can be greatly enhanced as has been impressively demonstrated by using multi-electrode detection equipment such as the CoulArray detector from ESA (model 5600 Chelmsford, MA, USA).[8] Here we describe a less sophisticated, but equally specific method to determine PB-DOPA using single electrode amperometric detection (model 460 Waters, Eschborn, Germany) and Tyr using fluorescence detection (model 470, Waters, Eschborn, Germany). We report human tissue-specific ratios of PB-DOPA/PB-Tyr in proteins with a molecular weight of more than 3 kDa. This ratio PB-DOPA/PB-Tyr is established in analogy to the well known ratio of 8-hydroxy-2′deoxyguanosine/2′-deoxyguanosine for the determination of oxidative stress-related DNA damage and should allow the comparison of oxidative stress-related protein damage in different organs, and should be of value to investigate ROS-mediated toxic actions following the treatment with pharmacotoxic agents.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased at the highest available analytical grade from Sigma (Deisenhofen, Germany) except for methanol, chloroform, and buffer salts that were purchased from Merck (Darmstadt, Germany), and neurotensin that was purchased from Bachem (Heidelberg, Germany). Laboratory gases of highest purity (nitrogen, argon) were delivered from local suppliers.

Equipment

Tissue homogenisation was achieved by sonication (sonifier 250 from Branson, Danbury, USA). Centrifuges used were: Minifuge 2 (Heraeus-Christ, Hanau, Germany); RC-5C (DuPont-Sorvall, Bad Homburg, Germany); and micro rapid K, (Hettich, Tuttlingen, Germany).

For lyophilisation of proteins reduced pressure was applied with an Univapo 150H connected to a cryo-unit Unicryo MC2L at −60 °C and a vacuum pump RC5 (Vacubrand, Wertheim, Germany). Gas-phase hydrolysis was done in a dessicator (Glaswerke Schott, Wertheim, Germany) under reduced pressure at about 15 mbar.

HPLC equipment for the separation and detection of PB-DOPA consisted of a pump type 300C (Gynkotek, Munich, Germany), rheodyne injector type 7125 (Latek, Eppenheim, Germany and Rheodyne Inc., Cotati, CA, USA), and an

electrochemical detector type 460 (Waters/Millipore, Eschborn, Germany) used at +0.75 V with settings filter 2, seconds 5, offset oxidation range 20 nA. Linearity was tested to be between 0.1 and 20 ng DOPA.

The determination of PB-Tyr used a fluorescence detector type 470 (Waters/Millipore, Eschborn, Germany). Excitation/emission wavelengths were set at 280/320 nm gain × 100, attenuation 4. Linearity for Tyr detection was determined to be at least between 50 and 250 ng.

Sample Preparation

In order to decrease the risk for artifactually formed or destroyed PB-DOPA by the presence of transition metals and to decrease the probability of contamination by free DOPA produced by *in vitro* oxidation of soluble Tyr to DOPA we decided to fractionate the tissue homogenate. With this procedure proteins with a molecular mass of more than 3 kDa have been investigated. Small peptides and free amino acids have not been investigated.

Isolation of Proteins > 3 kDa from Liver and Frontal Cortex

For quantitative acid hydrolysis, and in order to obtain unmodified isolates, proteins have to be essentially free of lipids, fatty acids and non-tightly bound transition metals. The frozen tissue (−70 °C) was homogenized with 9 µl / mg fresh tissue 150 mM ultrapure *o*-phosphoric acid containing 500 µM DTPA (*bis*(2-aminoethyl)-amine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid-Ca,3Na-salt, chelator for transition metals) by pulsed ultrasound under argon inert gas at 4 °C with 20 pulses for 500 ms duration each by means of a microtip. Approximately 200 µl homogenate was centrifuged at 45,000 g at 4 °C for 20 min to pellet the cell debris that was not disrupted by mild sonication. The supernatant containing hydrophilic proteins was transferred into a centricon tube molecular weight filter with a cut off at 3 kDa (centricon 3, Amicon, Witten, Germany). Peptides, free amino acids and salts were removed by centrifugation at 9,700 g, 4 °C for 15 min. The pellet, consisting of hydrophobic proteins and lipids, was rehomogenized twice with 100 µl water and centrifuged each time at 45,000 g at 4 °C for 20 min to remove residual hydrophilic proteins. The supernatants were transferred into the centricon tube of the first centrifugation step containing hydrophilic proteins. The pellet was then extracted twice with 200 µl chloroform/methanol (1/1, v/v) mixture containing 0.04 w/v% butylated hydroxytoluene as an antioxidant at 9,700 g, 4 °C for 15 min. The protein pellet was dried in a gentle stream of nitrogen. The lipid extracts were stored under argon at −70 °C for further analysis of fatty acids or lipid peroxidation products such as malonaldehyde or hydroxyalkenals described elsewhere.[7,8] The volume of the hydrophilic protein fraction is further reduced in centricon molecular weight filters at 9,700 g, 4 °C for 15 min and then back centrifuged to 1.5 ml tubes at 820 g, 4 °C. The resulting protein fraction is further dried by lyophilisation at reduced pressure.

Isolation of Hydrophilic Proteins from Red Blood Cells

20 µl of erythrocyte concentrate was obtained by centrifugation of EDTA-blood (EDTA monovettes, Sarstedt, Germany), and lysed with 120 µl lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA pH 7.4) at 4 °C for 15 min. After centrifugation at 360 g for 5 min at 4 °C the supernatant was transferred into molecular weight filters (Centricon 3, Amicon, Witten, Germany) cut off 3 kDa and again centrifuged at 4 °C at 9,700 g for 15 min. The pellet was rehomogenized twice with 100 µl water and each time centrifuged at 9,700 g at 4 °C for 15 min. The resulting supernatants were transferred into molecular weight filters and proteins were recovered after concentration to a volume of

20 µl from centricon tubes by back centrifugation, lyophilised and hydrolysed as described above.

Isolation of Hydrophobic Proteins from Red Blood Cells

500 µl of erythrocyte concentrate, obtained after centrifugation of EDTA-blood, was lysed with 3000 µl lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA pH 7.4) at 4 °C for 15 min. After centrifugation at 360 g for 5 min at 4 °C the supernatant was discarded and the pellet relysed with 1000 µl lysis buffer as described. The resulting pellet was rehomogenised with 500 µl water and centrifuged at 9,700 g at 4 °C for 15 min. Finally, the pellet was extracted twice with 500 µl chloroform/methanol (1/1, v/v) containing 0.04 w/v% butylated hydroxytoluene as an antioxidant (centrifugation at 45,000 g for 20 min at 4 °C). Chloroform and methanol were removed from the lipid extracts and the protein pellet in a gentle stream of nitrogen. The lipids can be stored under argon at −70 °C for further analysis. The protein pellet was hydrolysed as described below.

Gas-phase Acid Hydrolysis of Dried Protein Pellets

The method based on the hydrolysis procedure described by Gieseg *et al.*,[1] was modified. Up to 12 protein samples, dry pure BSA or dry neurotensin standard in plastic cups were hydrolysed in a dessicator containing two levels. Protein samples stood on a platform inside the dessicator, with the hydrolysing agent on the lower level. The glas dessicator was flushed for 10 min with argon to remove oxygen and a low pressure was created using a water pump for 5 min at about 15 mbar. For the prevention of DOPA oxidation the hydrolysing agent contained 2 ml 6 N HCl, 1% w/v phenol and 100 µl thioglycolic acid. Hydrolysis was quantitative after 16 h at 110 °C in an oven. After cooling the dessicator the samples were diluted in 200 µl 0.3 N HCl.

To determine the recovery of DOPA and Tyr during hydrolysis 10 ng pure DOPA or 10 µg pure Tyr were hydrolysed. The recoveries were 90% for DOPA ($n = 4$) and almost 100% for Tyr ($n = 4$). We also tested the recovery of DOPA and Tyr in the presence of proteins isolated from tissue. For this purpose 20 ng DOPA or 20 µg Tyr were added to the fraction of membrane proteins. For the determination of the recovery of proteins from the hydrophilic fraction, 50 ng of DOPA or 50 µg of Tyr were added. The overall recoveries after hydrolization were close to 100% for DOPA in both fractions and 96% for Tyr in the hydrophilic fraction and 90% in the fraction of the membrane proteins ($n = 4$). In all experiments made with human samples or BSA we determined the recovery for DOPA and used this individual result for the calculation of DOPA content in the samples. To determine the effectiveness of the gas phase hydrolysis 200 µg of neurotensin Gln4 (MW 1672 g/mol), a peptide of 13 amino acids with two of them being Tyr, and 200 µg bovine serum albumin (BSA, MW 69,293 g/mol), a protein consisting of 607 amino acids with 20 of them being Tyr were hydrolised. The amount of released Tyr was determined. The level of hydrolysis of neurotensin Gln4 reached 94% ($n = 4$), and that of BSA was 95% ($n = 4$).

Liquid Chromatography and Detection

For the determination of PB-Tyr and DOPA a total volume of 20 µl in 0.3 N HCl was injected into the rheodyne 6 valve injector. Separation of Tyr and DOPA was carried out at room temperature on a 5 µm, 250 × 4.0 mm Nucleosil C18 reversed phase column (Macherey & Nagel, Düren, Germany). The mobile phase consisted of 150 mg/l pentane sulfonic acid, 50 mg/l disodium EDTA, 1 ml/l diethylamine in water adjusted to pH 2.2 with ultrapure *o*-phosphoric acid. The flow rate was set at 1.0 ml/min. The

two detectors were connected in series with first detecting Tyr. Detection limit for DOPA was 0.1 ng corresponding to 0.5 pmol per injection. Detection limit for Tyr was 50 ng corresponding to about 0.3 nmol. Identification and quantitation of peaks was achieved by comparing peak heights and retention times with the respective peak characteristics of commercial standards (Sigma, Deisenhofen, Germany).

RESULTS

Following pilot experiments (not shown) aimed at optimizing tissue sample protein isolation, hydrolysis, and determination we were able to detect DOPA at concentrations as low as 0.1 ng (about 0.5 pmol) from pure BSA and from tissue. The resulting chromatograms of DOPA and Tyr of a representative HPLC analysis from frontal cortex protein extracts are given in Figure 1. As depicted, there is no interference from other compounds. Electrochemical detection confers sensitivity and a rather high specificity for catechol compounds. Another factor for high specificity is the fact that cations (DOPA is protonated at pH 2.2) are selected by forming ion pairs with pentane sulfonic acid in the mobile phase. Tyr exists in high amounts in the protein hydrolysate and thus can be measured by fluorescence spectrometry without sensitivity problems. The mobile phase is optimized as well for good separation of Tyr from other fluorescing compounds. Theoretically BSA that contains 20 tyrosines per molecule should not exhibit DOPA. Techniques involving sample treatment with chemicals that contain traces of metals might, however, catalyse adverse reactions eventually leading to oxidation of Tyr to DOPA and even so-called pure BSA purchased might contain trace amounts of DOPA. Thus, in order to test the background levels of DOPA we measured the basal content of Tyr and DOPA in pure BSA (MW 69,293 g/mol with

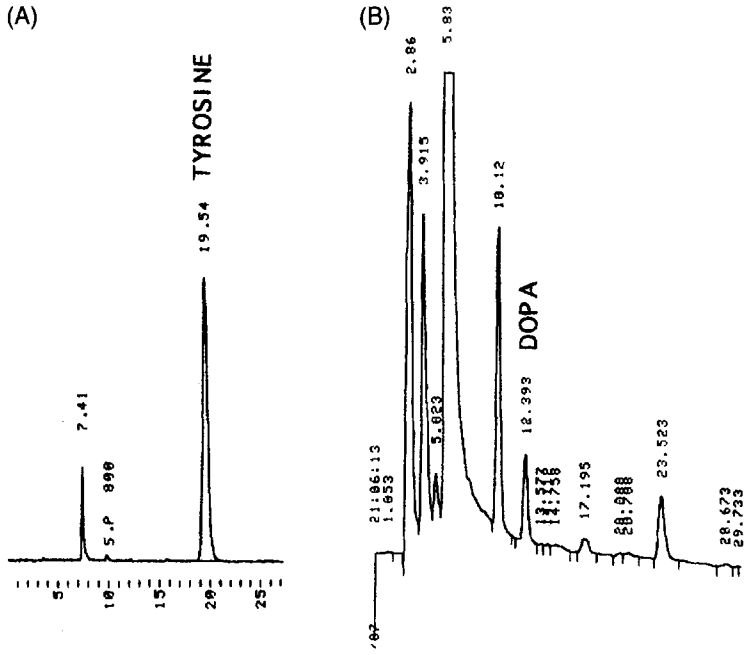

FIGURE 1 Chromatograms of Tyr (A) and DOPA (B) of a representative sample isolated from frontal cortex. HPLC conditions are stated in the experimental section. Retention times are given as min.

TABLE I Contents of 3,4-dihydroxyphenylalanine (DOPA) and tyrosine (Tyr) as well as DOPA/Tyr ratios in human frontal cortex, liver, and red blood cells

	ng/mg W.Wt Brain	ng/mg W.Wt Liver	ng/10^9 Erythrocytes
DOPA in membrane proteins	0.52 ± 0.12	0.63 ± 0.06	0.29 ± 0.05
DOPA in hydrophilic fraction	0.36 ± 0.01	0.41 ± 0.10	260,72 ± 17.39
Total DOPA	0.88 ± 0.12	1.04 ± 0.11	261.01 ± 17.43
Tyr in membrane proteins	355 ± 36	513 ± 62	431 ± 63
Tyr in hydrophilic fraction	1636 ± 155	1941 ± 212	786,954 ± 52,846
Total Tyr	1992 ± 141	2455 ± 267	787,385 ± 52,808
	ratio × 10^{-4}	ratio × 10^{-4}	ratio × 10^{-4}
DOPA/Tyr in membrane proteins	13.1 ± 2.4	12.1 ± 2.3	6.2 ± 0.7
DOPA/Tyr in hydrophilic fraction	2.0 ± 0.2	2.1 ± 0.7	3.1 ± 0.4
Total DOPA/Tyr	4.1 ± 0.7	4.1 ± 0.9	3.1 ± 0.4

Data are expressed as means (ng/mg wet weight) ± SEM of four independent determinations from different individuals processed according to the procedures described in the experimental section.

5.2% Tyr mass content). BSA contained a similar ratio of DOPA/Tyr as that extracted from the hydrophilic fraction of frontal cortex tissue (Table I).

In our experiments levels of DOPA in pure BSA never exceeded 10 ng/mg BSA. This corresponds to one molecule of PB-DOPA per 7000 Tyr or 285 molecules of BSA giving a ratio of DOPA/Tyr of approximately 1.8×10^{-4} in BSA. When BSA was incubated with increasing concentrations of ferrous sulfate (10–100 µM) prior to lyophilisation, levels of DOPA increased dose-dependently (Figure 2). Incubation of BSA with 100 µM ferrous sulfate for 180 min increased the DOPA/Tyr ratio three times. This result emphasizes that iron may critically interact with PB-Tyr *in vitro* giving rise to artifactual DOPA formation if care is not taken to remove soluble iron from tissue samples. Thus, we homogenized tissue samples in the presence of an excess of the metal chelator (DTPA) and washed all our protein pellets twice with bidistilled water in order to remove most of the non-tightly bound transition metals from protein samples. The inter-assay variability for DOPA on the same sample was in the range of 7–15% and that for Tyr ranged 4–7%.

The inter-individual variance in physiological levels of PB-DOPA/Tyr ratios was determined to range between 20% and 40% (frontal cortex controls $n = 10$). Comparing the content of DOPA and Tyr of frontal cortex, liver and red blood cells, reveals significant differences in background hydroxylation levels of Tyr in these tissues (Table I). In liver and frontal cortex ratios of DOPA/Tyr are quite similar. Red blood cells, however, contain in the fraction of the hydrophobic proteins about half the amount of DOPA per Tyr as compared to liver and frontal cortex

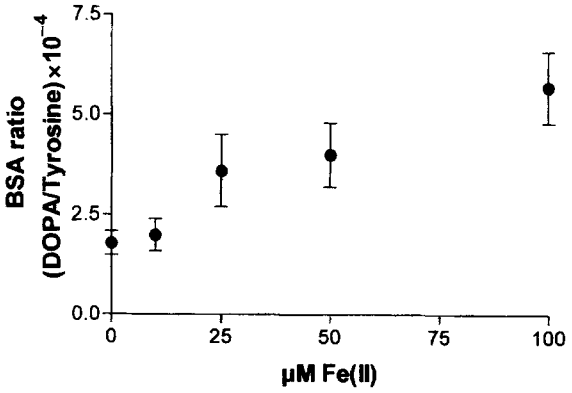

FIGURE 2 Iron-dependent stimulation of Tyr oxidation to DOPA in BSA. Data are given as mean ± SE ng/mg BSA of four independent determinations. 1 mg/ml BSA in 10 mM phosphate buffer pH 7.4 were incubated with increasing concentrations of ferrous sulfate at 37 °C for 180 min. 200 µl of the sample were transferred into molecular weight filters cut off 3 kDa and centrifuged at 9,700 g, 4 °C for 15 min. The samples were washed twice with 200 µl water to remove residual iron. The protein residue was lyophilised and analysed as mentioned in the experimental section.

from humans. All tissue values in the fraction of the hydrophobic proteins are about six times higher than pure BSA. In the hydrophilic protein fraction levels of DOPA/Tyr ratios are similar to those found in BSA without stimulation by iron.

DISCUSSION

Here we present a sensitive method for the measurement of the tissue Tyr hydroxylation ratio DOPA/Tyr. The procedure of gas-phase hydrolysis has several advantages compared with other methods for protein hydrolysis. From our protein isolation procedure the proteins are obtained comparably clean and dried. The hydrolysis takes place in hydrochloric acid vapour which contacts the dried proteins without undue wettness. Thus, the resulting amino acids are very concentrated, contain insufficient acid to disturb further analysis and can be diluted in very small amounts of solvent, enabling the analysis of very small amounts of DOPA. Using phenol and thioglycolic acid in a nearly anoxic environment prevents artifactual Tyr oxidation. Other procedures using enzymatic protein degradation or using toluene sulfonic acid as hydrolysing agent are not recommended because these methods often do not quantitatively digest proteins (data not shown). We did however, not utilize an alternative method that has been presented using the "pronase" class of nonspecific proteases isolated from *Staphylococcus griseus*.[8,11]

It has to be emphasized that proteins have to be semipurified, lipids, fatty acids, and transition metals removed as described above before lyophilisation in order to give reproducible hydrolysis without altering the amount of Tyr and DOPA. This has been emphasized as well recently by Davies *et al.*[12] summarizing in an excellent review the precautions to obey if stable markers of damage to proteins are to be determined.

The background levels of DOPA in BSA standards might be in principle explained by a transition metal-catalyzed oxidation of Tyr as shown by the oxidation of BSA *in vitro* by 100 μM ferrous iron. The total DOPA/Tyr ratios reported here from brain and liver are both about 4 DOPA/10,000 Tyr and in red blood cells we found about 3 DOPA/10,000 Tyr. These levels are even below the levels reported under physiological conditions for oxidized LDL protein which contain 6 DOPA per 10,000 Tyr under non-pathological conditions. In contrast, ratios increased to 14 DOPA per 10,000 Tyr in advanced atherosclerotic plaques[13] or even up to 598 DOPA/10,000 Tyr in type IV cataract vs 51/10,000 in normal human lens.[14]

Interestingly, DOPA/Tyr ratios of water-insoluble hydrophobic proteins from membranes are about six fold higher than the ratios found in the hydrophilic fractions from frontal cortex and liver. This difference might be explained by increased susceptibility for oxidation of lipophilic proteins in the course of lipid peroxidation processes. It might be possible as well, that because of a longer average half-life of membrane proteins DOPA might accumulate to a certain extent, or that hydroxylation in membranes might be facilitated by electron transfer reactions involving cytochromes in the endoplasmic reticulum or the mitochondrial inner membrane.

HPLC-electrochemical detection is emerging as the technique of choice for sensitive and specific detection of DOPA.[12] If care is taken to prevent artifactual oxidation of PB-Tyr, PB-DOPA can be considered as a marker of tissue oxidative stress. The absolute levels of PB-DOPA resulting exclusively from hydroxyl radical attack however, are still hard to quantitate because DOPA can be generated either via peroxyl radical chemistry or disproportionation reactions without the participation of reactive oxygen species.[15] In addition the considerable inter-individual differences from human tissues, which might originate as well in part from different cell types due to unprecise dissection

of tissue, requires a reasonable sample size to elucidate differences of DOPA/Tyr ratios between distinct nosological entities.

Nevertheless, we believe that the described method may be very useful in determining protein hydroxylation in tissues vulnerable to oxidation such as red blood cells, brain tissue and liver if prepurification of proteins prior to hydrolysis by removing lipids, fatty acids and salts is performed, hydrolysis takes place under reductive conditions in the absence of oxygen in hydrochloric acid vapour, and if a CoulArray detector is not available.

In conclusion, the establishment of DOPA/Tyr tissue ratios by simultaneous measurement of DOPA and Tyr within the same sample should make this method valuable to compare oxidative stress to proteins occurring in different tissues and species because it is independent of the amount and type of protein analysed whilst only dependent on the overall amount of Tyr in the tissue. This should facilitate the evaluation of the relevance of protein hydroxylation in a given disease or experimental model in comparison to adequate controls.

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