Extensive Heterogeneity of Human Urokinase, As Detected by Two-Dimensional Mapping

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Urokinase (uPA, urinary plasminogen activator) is a serine protease belonging to the peptidase S1 family and specifically cleaving the zymogen plasminogen into the active form, plasmin, which, in turn, degrades the fibrin clots. For this property, uPA is widely used as fibrinolytic agent in thrombolytic therapy. Urokinase is used clinically as a thrombolytic agent in the treatment of severe or massive deep venous thrombosis, pulmonary embolism, myocardial infarction, and occluded intravenous or dialysis cannulas.[1−3] It is also administered intrapleurally to improve the drainage of complicated pleural effusions and empyemas. It is the most effective drug in myocardial infarction.[4,5] The enzyme was originally identified in human urine for its ability to catalyze the transformation of plasminogen into its active form, plasmin, which degrades fibrin and extracellular matrix components. The binding of uPA to its specific surface receptor (uPAR) amplifies the cell-surface plasminogen activation, thus enhancing pericellular proteolysis.

Urokinase is a two-chain glycoprotein containing 411 amino acids with 12 disulfide bonds; it is present in human urine (from which it is commonly purified[6,7]) in two active forms: high molecular-mass urokinase (HMr uPA) and low-molecular-mass urokinase. HMr uPA consists of two polypeptide chains: long chain A (residues 21−177) and chain B (residues 179−431), connected by an interchain disulfide bridge (AA 168 and AA 299). LMr uPA contains the entire chain B and a short fragment (residues 156−177) of chain A.[8]

Several approved products are commercially available; the one adopted and used has been produced by Syner-med (London, U.K.). At Bio API, a new product has been prepared (also purified from human urine) and its physicochemical properties have been compared with the Syner-med reference standard. The present report involves the analysis and characterization of this preparation and describes an extensive heterogeneity of both the Syner-med reference standard and the Bio API enzyme, as revealed by two-dimensional (2D) map analysis.

EXPERIMENTAL SECTION

Chemicals and Reagents. Laemmli buffer, 40% acrylamide/bis solution, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), molecular mass standards, 4%−12% precast gels and immobilized pH gradient (IPG, pH 3−10 linear) gel strips were obtained from Bio-Rad Laboratories (Hercules, CA). β-mercaptoethanol, dithiothreitol (DTT), ammonium persulfate, 3-[3-cholamidopropyl dimethylammonio]-1-propanosulfonate (CHAPS), acetonitrile (ACN), trifluoroacetic acid (TFA), sodium dodecyl sulfate (SDS), formic acid (FA), De-Streak [bis(2-hydroxyethyl)disulfide, $(HOCH_2CH_2)_2S_2$)], TCEP [tris-(2-carboxyethyl)phosphine hydrochloride], and all other

chemicals used throughout the experimental work were current, pure, analytical-grade products and purchased from Sigma–Aldrich (St. Louis, MO). Three batches of purified urokinase (1301, 1302, 1303) were obtained from Bio API (Active Pharmaceutical Ingredients), and commercial urokinase was purchased from Syner-med (London, U.K.).

SDS–Polyacrylamide Gel Electrophoresis (SDS-PAGE). Forty micrograms (40 μg) of different samples, in the presence of reducing Laemmli[9] buffer (4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.004% Bromophenol Blue, and 0.125 M Tris HCl, pH ~6.8), or nonreducing Laemmli buffer (4% SDS, 20% glycerol, 0.004% Bromophenol Blue, and 0.125 M Tris HCl, pH ~6.8) were loaded onto the precast gel. The cathodic and anodic compartments were filled with tris-glycine buffer (pH 8.3), containing 0.1% SDS. Electrophoresis was at 100 V until the dye front reached the bottom of the gel. Staining and distaining were performed with Colloidal Coomassie Blue and 7% acetic acid in water, respectively. The SDS-PAGE gels were scanned with a VersaDoc imaging system (Bio-Rad). The digitized images were further analyzed using the Quantity One (Bio-Rad) software.

Two-Dimensional Electrophoresis Analysis. For 2D mapping, the desired volume of each sample was solubilized in the "2-D sample buffer" (7 M urea, 2 M thiourea, 3% CHAPS, 20 mM Tris) to a final concentration of 2 mg/mL protein, and the disulfide bridge reduction was allowed to proceed at room temperature for 60 min via the addition of TCEP at a final concentration of 5 mM. For alkylating the reduced –SH groups, 150 mM De-Streak (diluted directly from the stock 8.175 M) was added to the solution, followed by 0.5% pH 3–10 Pharmalyte (diluted directly from the stock, 40% solution) and a trace amount of Bromophenol Blue. IPG strips (7 cm long, pH 3–10 linear)[10] were rehydrated with 150 μL of protein solution for 4 h. Isoelectric focusing (IEF) was carried out with a Protean IEF Cell (Bio-Rad Laboratories) in a linear voltage gradient from 100 V to 1000 V for 5 h, at 1000 V for 4 h, followed by an exponential gradient up to 5000 V, for a total of 25 kV/h. For the second dimension, the IPGs strips were equilibrated for 25 min in a solution containing 6 M urea, 2% SDS, 20% glycerol, 375 mM Tris-HCl (pH 8.8) under gentle shaking. The IPG strips were then laid on an 8%–18% acrylamide gradient SDS-PAGE gel slab with 0.5% agarose in the cathodic buffer (192 mM glycine, 0.1% SDS, and Tris-HCl, to pH 8.3). The electrophoretic run was at 5 mA/gel for 1 h, followed by 10 mA/gel for 1 h and 15 mA/gel until the dye front reached the gel bottom. Gels were incubated in a colloidal Coomassie Blue,[11] solution and destaining was performed in 7% acetic acid until clear background was obtained, followed by a rinse in pure water. A spot count was performed directly after drying of the two-dimensional (2D) plates. The various maps were compared via the PDQuest software (version 9.1) from Bio-Rad Laboratories.

Mass Spectrometry and Data Analysis. The sample lanes of SDS PAGE or the spots from 2D gels were cut and proteins were reduced by 10 mM DTT, alkylated by 55 mM iodacetamide and digested with 1 ng/μL trypsin in 25 mM ammonium bicarbonate at 37 °C overnight. The tryptic mixtures were acidified with formic acid up to a final concentration of 10%. Eight microliters (8 μL) of tryptic digest for each band were injected in a nanochromatographic system (UltiMate 3000 RSLCnano System, Thermo Scientific). The peptide mixtures were loaded on a reverse-phase trap column (Acclaim PepMap100, C18, 100 Å, 100 μm i.d. × 2 cm, Thermo Scientific) for the cleanup and preconcentration. After cleanup, the valve was switched to place the trap column in series with a fused-silica reverse-phase column (picoFrit column, C18, 2.7 μm, New Objective). The peptides were eluted with a 30 min gradient from 4% buffer A (2% acetonitrile and 0.1% formic acid in water) to 60% buffer B (2% water and 0.1% formic acid in acetonitrile) at a constant flow rate of 300 nL/min. The liquid chromatograph was connected to a mass spectrometer (Model LTQ-XL, Thermo Scientific) equipped with a nanospray ion source. Full scan mass spectra were acquired in the mass range m/z 350 to m/z 2000 Da and the five most intense ions were automatically selected and fragmented in the ion trap. Target ions already selected for mass spectrometry (MS/MS) were dynamically excluded for 30 s. The MS data were analyzed separately by the Mascot search engine (version 2.3.01), using Proteome Discover software (v. 1.2.0 Thermo) and consulting Swiss-Prot protein database without taxonomy restriction (all entries). Oxidation of methionine residues

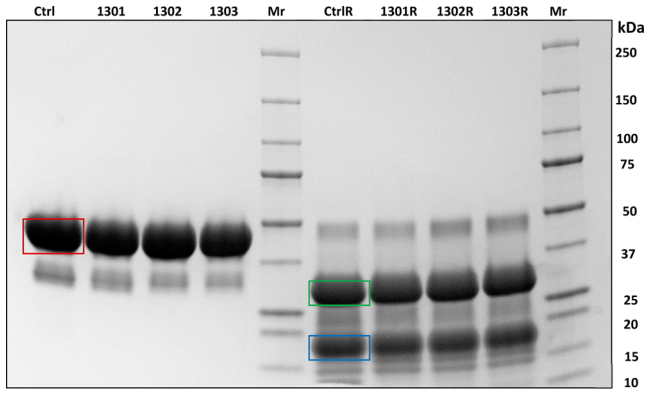

Figure 1. SDS-PAGE of nonreduced (left side) and reduced (R, right side) purified urokinase. "Ctrl" denotes the control, which is the Syner-med reference standard; "1301", "1302", and "1303" represent the three batches of Bio APi urokinase. Red box indicates the native, nonreduced enzyme; green box indicates urokinase chain B; and blue box indicates urokinase chain A. Total load = 40 μg protein; staining obtained using micellar Coomassie Blue. "Mr" denotes molecular mass standards.

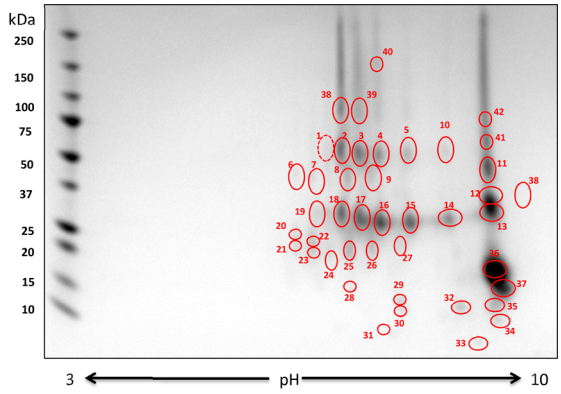

Figure 2. Two-dimensional map (2D) of a 1:1 mixture of Syner-med reference standard and Bio API urokinase. The 42 visible spots have been circled in red, excised, digested, and analyzed via MS with an LTQ-XL mass spectrometer. The identifications are shown in Table 1. First dimension: IPG 3–10 linear pH gradients; second dimension: 8%–18% acrylamide porosity gradient. Total sample load: 120 μg protein; staining: micellar Coomassie Blue. The vertical lane on the left gel side contains the molecular mass standards (Mr ladder).

Table 1. MS Analysis of the 42 Spots Excised from the 2D Map of Figure 2

N spot	accession number	protein name	Mascot score	Mr	N peptides
1	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	106	49901	6
2	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	158	49901	2
2	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	158	49901	2
3	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	187	49901	8
4	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	486	49901	10
4	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	486	49901	10
5	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	314	49901	8
6	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	156	49901	4
6	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	156	49901	4
7	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	171	49901	5
9	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	167	49901	6
10	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	116	49901	5
10	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	116	49901	5
11	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	706	49901	8
12	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	495	49901	10
12	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	495	49901	10
13	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	489	49901	9
14	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	672	49901	13
15	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	1482	49901	16
16	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	992	49901	15
17	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	1227	49901	13
18	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	753	49901	12
20	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	102	49901	9
21	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	163	49901	6
23	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	228	49901	6
24	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	118	49901	6
25	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	261	49901	8
26	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	99	49901	8
27	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	215	49901	9
28	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	320	49901	9
29	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	135	49901	8
30	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	168	49901	6
31	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	116	49901	4
32	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	82	49901	4
34	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	294	49901	8
35	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	263	49901	8
36	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	479	49901	13
37	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	266	49901	6
38	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	409	49901	10
39	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	250	49901	8
40	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	111	49901	7
41	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	679	49901	12
42	UROK_HUMAN	Urokinase-type plasminogen activator OS = *Homo sapiens*	406	49901	13

was set as variable modification; two missed cleavages were attributed to trypsin; peptide mass tolerance was set to 1 Da, fragment mass tolerance to 0.8 Da, and an ion source cutoff of 20 was chosen. The false discovery rate, as determined using the Proteome Discoverer, obtained by consulting the Mascot decoy database, was <0.01. Two biological replicates and three technical replicates were performed for each batch.

RESULTS AND DISCUSSION

To start with, the different batches were analyzed by mono dimensional SDS PAGE. All Bio API samples and the Syner-med specimen showed the same profile (see Figure 1). As visible in the SDS PAGE tracks under nonreducing conditions (four lanes on the left gel side), there are two forms of uPA: a major high-M_r component (~50 kDa) and a minor one (~30 kDa), representing ca. 14% in the Syner-med sample and 11% in the Bio API sample, respectively (as assessed by profiling the lanes via the Quantity One software). Under reducing conditions, the first form containing the disulfide bridge is split into two heavy bands, chain B (25−37 kDa) and long chain A (20 kDa); the second one is reduced to chain B and short chain A (not visible on the gel). The large catalytic region (residues 179−424) and the N-terminal region are spaced by the "connecting peptide" (residues 152−177). The amino acids 34−57 region binds the uPA receptor; uPA is phosphorylated at serine residues 158 and 323, while the AA 38 is fucosylated.[12,13] The three samples of Bio API urokinases are in very good agreement with the relative SDS-PAGE profiles exhibited by the commercial Syner-med enzyme under both nonreducing and reducing conditions (see Figure 1). Thus, these data confirm the quality of the Bio API urokinase in accordance with the Syner-med sample without the presence of

any contaminants or degradation products during the processing stages.

The different batches were then analyzed by 2D PAGE, which, in contrast, displayed a very marked heterogeneity in both samples. The Syner-med specimen was analyzed (in triplicate) by 2D mapping, whereas two replicas were obtained for each of the three Bio Api samples. The specimens were run individually in 2D maps, which were then superimposed via the PDQuest software and finally they were also run in a 1:1 mixture. All samples showed, consistently, 40−42 spots, spread in the pI range from 6.5 to 9.5 and in the Mr interval of 10−50 kDa. Figure 2 shows a representative 2D gel of the 1:1 mixture run in an IPG 3−10 pH interval in the first dimension and in a 8%−18% T porosity gradient in the second dimension gel. The 42 bands detected (circled in red) were excised, eluted, digested, and submitted to MS analysis via nLC MS/MS (Model Ultimate 3000, coupled with Model LTQ XL, Thermo Scientific) and identified by Mascot by consulting the Uniprot_human protein database.[14] All protein spots were identified as Urokinase plasminogen activator, as shown in Table 1.

In a way, such an extensive heterogeneity for a human sample (especially if purified from a biological fluid) is not unexpected. For instance, already in 2003, Pieper et al.,[15] after extensive chromatographic separation of human serum proteins, could display, in large 2D gels, ∼3700 polypeptide zones, which were all subjected to MS analysis. Yet, this vast number of spots yielded only 325 unique gene products, which means that, on average, each individual serum protein was further processed/modified to generate ∼12 spots. Similar results were also obtained in the analysis of prion protein, as isolated and purified from human cerebrospinal fluid or (post-mortem) from the cerebellum of several normal individuals and from patients suffering from sporadic Creutzfeldt−Jakob disease. Typically the prion protein is resolved into three bands in SDS-PAGE, corresponding to the nonglycosylated, monoglycosylated, and diglycosylated forms. Yet, when run in 2D maps, the three zones are resolved into no fewer than 64 spots.[16,17] This situation if further aggravated when purifying proteins from urine, since in this biological fluid proteases are constantly present and quite active.[18] This is why 2D map analysis coupled to MS identification of excised spots is quite important in order to assess if such heterogeneity is due to post-synthetic modifications of the desired product or to the presence of impurities in the samples under analysis.

A note of caution: Reduction and alkylation of −SH groups should be performed prior to entering the electric field, i.e., also prior to the first-dimension isoelectric focusing run. Failure to do so would result in a series of spurious spots, because of the formation of homo-oligomers and hetero-oligomers in the absence of alkylation, which is a phenomenon amplified especially with basic pI proteins, as in the case of urokinase.[19−21] Thus, here, correctly, urokinase was reduced and alkylated prior to 2D mapping. The next question to be determined is which one is the best alkylating agent. Although most investigators still use the very old protocol of iodoacetamide addition, this reagent is quite poor and rarely can achieve better than 60% alkylation, especially in the presence of surfactants.[20,21] By far the best alkylating agent, able to drive the reaction to completion, even under the most adverse conditions, is 4-vinylpyridine.[21,22] Yet, even this unique chemical is rarely used today, because of its volatility and toxicity. The next best, then, is the method of Olsson et al.,[23−25] who have proposed the oxidation of thiol groups in proteins to mixed disulfides, by using, in the gel rehydration solution, just prior to the focusing process, an excess (150 mM) of hydroxyethyldisulfide ($HO-CH_2-CH_2-S-S-CH_2-CH_2-OH$), which will act as an efficient blocking agent of reduced −SH groups. This was the method finally adopted in our urokinase treatment.

CONCLUSIONS

Our data have fully validated the Bio API product and shown that, in comparison with the Syner-med urokinase reference standard, it displays identical polydispersity and contains only the desired enzyme in different isoforms and with post-translational modifications and fragments. The present data also underline the necessity, when analyzing samples from biological fluids, to perform two-dimensional mapping and MS analyses of each individual spot, to confirm their identity and exclude the presence of nonrelated impurities.

Notes

The authors declare no competing financial interest.

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