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15

Proteomics Analysis of Kinetically Stable Proteins

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1. Introduction

The term "kinetic stability" (KS) is sometimes used to describe proteins that are conformationally trapped by the presence of an unusually high-energy unfolding barrier that considerably decreases their unfolding rate under various conditions. This barrier allows kinetically stable proteins (KSPs) to maintain their fold and activity over longer periods, even in inhospitable environments. KS is likely to play important biological roles, such as the regulation of protein turnover, protection from proteolytic degradation, and blocking access to aggregation-prone conformations. However, the chemical-physical basis and the diversity of biological-pathological roles of protein KS remain poorly understood, in part because for many years the study of KS was limited to individual pure proteins, and involved spectroscopic instrumentation that was not available to most researchers. In this chapter, we will review the discovery of a correlation between a protein's KS and its resistance to the detergent sodium dodecyl sulfate (SDS), and the subsequent development of a diagonal two-dimensional (D2D) SDS-PAGE assay and capillary electrophoresis approaches to identify the proteome of KSPs in any cell or organism.

1.1 Thermodynamics vs kinetic stability

The concept of kinetic stability (KS) as an alternative explanation for protein stability, independent from thermodynamic stability, was introduced in the early 90's (Fig. 1) (Baker & Agard, 1994; Baker, Sohl, & Agard, 1992). KS is conveniently explained by illustrating the unfolding process as an equilibrium reaction between the native folded state (N) and the unfolded state (U), separated by a transition state (TS) (Fig. 1). Since the height of the TS free energy determines the rate of folding and unfolding, the unusually high unfolding free energy barrier of a KSP results in a very slow unfolding rate that practically traps the protein in its native state (Fig 1). It has been suggested that the existence of a high energy barrier separating the folded and unfolded states is an evolutionary feature to preserve protein activity in the severe conditions they might encounter in nature (Cunningham, Jaswal, Sohl, & Agard, 1999). This is consistent with the observation that thermodynamic stability by itself does not fully protect proteins from irreversible denaturation and aggregation arising from denatured conformations that fleetingly form under physiological

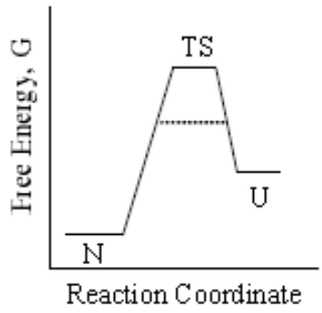


Fig. 1. Free energy diagram illustrating the higher unfolding energy barrier for a kinetically stable protein under native conditions, as compared to that of a normal protein (represented by the dash line). The labels represent the native (N) state, unfolded (U) state, and transition state (TS). Reprinted with permission from (Manning, M., & Colón, W. (2004). Structural basis of protein kinetic stability: Resistance to sodium dodecyl sulfate suggests a central role for rigidity and a bias towards beta sheet structure. *Biochemistry, 43*, 11248-11254). Copyright (2004) American Chemical Society.

conditions (Plaza del Pino, Ibarra-Molero, & Sanchez-Ruiz, 2000). Thus, the presence of an unfolding TS with high energy may protect susceptible proteins against harmful conformations. In summary, KSPs are basically slow-unfolding proteins that are more resistant to aggregation and degradation.

Protein misfolding diseases (PMD) (Johnson, et al., 2005), include some of the most common human ailments, including Alzheimer's, Parkinson's, Type II diabetes, and cancer (Dobson, 2001; Stefani & Dobson, 2003). There is strong evidence that loss or gain of SDS-resistance (correlating to KS), facilitated by mutation, protein damage, or a compromised quality-control system, is linked to some PMD. Aging appears to also play a role, consistent with the late-onset of most PMD. The loss of KS might represent a hazard for the organism, especially for older individuals who have less efficient protein quality control systems (Koga, Kaushik, & Cuervo, 2010; Luce, Weil, & Osiewacz, 2010). In familial amyloid polyneuropathy it is known that missense mutations can compromise the KS of transthyretin (TTR), facilitating tetrameric TTR dissociation and subsequent aggregation into amyloid fibrils (Saraiva, 1995). Remarkably, native state kinetic stabilization of TTR via several strategies (Hammarstrom, Schneider, & Kelly, 2001) can restore the KS of mutated TTR, and is emerging as a therapeutic strategy for TTR amyloidosis (Johnson, et al., 2005).

It is also plausible that some diseases might be associated with protein misfolding into a toxic species with high KS, since such a species would be more difficult to degrade. A striking example is the prion protein, which is linked to various genetic and transmissible diseases (Horwich & Weissman, 1997). The native prion protein lacks KS (Hornemann & Glockshuber, 1998), but the misfolded infectious prion has high KS (Prusiner, Groth, Serban, Stahl, & Gabizon, 1993), thus explaining why it survives the GI track in transmissible prion diseases. Furthermore, the abnormal *in vivo* presence of SDS-resistant (i.e. kinetically stable – see section 3 for SDS-KS correlation) and potentially toxic species is a feature of various PMD, including Alzheimer's disease and Parkinson's disease (Cappai, et al., 2005; Enya, et al., 1999; Funato, Enya, Yoshimura, Morishima-Kawashima, & Ihara, 1999; Haass & Selkoe,

2007; Kawarabayashi, et al., 2004; Lee, et al., 2011; Lesne, et al., 2006; McLean, et al., 1999; Podlisny, et al., 1995; Roher, et al., 1996).

1.2 SDS as a probe for protein kinetic stability

SDS-PAGE was introduced in the 1960s as a method for separating proteins (Shapiro, Vinuela, & Maizel, 1967). Currently SDS-PAGE is perhaps the most fundamental technique in protein biochemistry. The interaction between a protein and SDS is complex and involves nonpolar and electrostatic interactions. In spite of the ubiquitous use of SDS, it is still poorly understood how it denatures proteins when present at above its critical micelle concentration (CMC) (Otzen, 2002). It has been suggested that at concentrations less than 100 mM, (CMC of SDS is ~7 mM in water (Reynolds, Herbert, Polet, & Steinhardt, 1967)) SDS denatures proteins by a mechanism involving ligand-binding-type unfolding kinetics. Furthermore, it was shown that SDS does not alter the transition state energy for protein unfolding (Otzen, 2002), thereby implying that SDS's interaction with a protein's surface has minor effect on the structure and free energy of its native state (Otzen, 2002).

In 2004, we demonstrated a correlation between KS and the resistance of proteins to denaturation by SDS, resulting in a simple assay that is very effective for probing the KS of proteins (Manning & Colón, 2004). The initial step of our study involved the identification of SDS resistance from a group of 33 proteins. SDS resistance was assayed by comparing the migration on a gel of boiled and unboiled protein samples containing SDS (Fig. 2). Proteins that migrated to the same location on the gel regardless of whether the sample was boiled were classified as not being stable to SDS (Fig. 2B). Those proteins that exhibited a slower migration when the sample was not boiled were classified as being resistant to SDS-induced denaturation (Fig. 2A). The slower migration is a sign of less SDS binding and thereby of a lesser overall negative charge of the SDS-protein complex compared to the fully SDS-bound proteins. Of the proteins tested, eight were found or confirmed to exhibit resistance to SDS, including superoxide dismutase (SOD), streptavidin (SVD), TTR, P22 tailspike protein (TSP), chymopapain (CPAP), papain (PAP), avidin (AVD), and serum amyloid P (SAP) (Fig. 2A).

To probe the KS of our SDS-resistant proteins, we used fluorescence spectroscopy and demonstrated their slow unfolding rates even in 6.6 M guanidine hydrochloride (GuHCl) at 20 °C. To gather further evidence of the KS exhibited by these proteins under native conditions, their unfolding rate constants in the absence of the denaturant were obtained by measuring the unfolding rate at different GuHCl concentrations and extrapolating to 0 M. The native state unfolding rate constants for TTR (Lai, McCulloch, Lashuel, & Kelly, 1997) and SVD (Kurzban, Bayer, Wilchek, & Horowitz, 1991) were obtained from the literature. The unfolding rate in the absence of denaturants for all of the SDS-resistant proteins was found to be very slow (Table 1), with protein half- lives ranging from 79 days to 270 years.

The observation that all of the SDS-resistant proteins were also kinetically stable, suggested that SDS resistance might be caused by KS. To further test the correlation between KS and SDS resistance, we selected a group of six proteins that did not exhibit resistance to SDS and analyzed their unfolding behavior in varying concentrations of GuHCl. The group was chosen to represent a variety of structural characteristics. At 6.6 M, the unfolding of these proteins was too fast to detect with a standard fluorescence spectrophotometer. The lack of

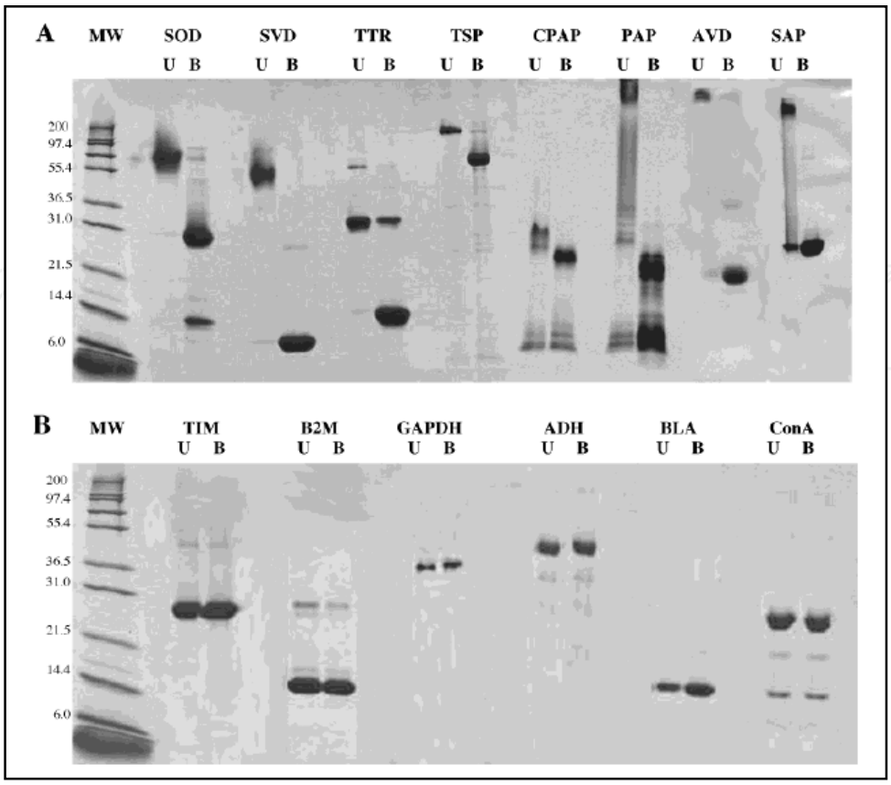


Fig. 2. SDS-PAGE assay to identify SDS-resistant proteins (Manning & Colón, 2004). We tested the proteins (A) papain (PAP), chymopapain (CPAP), avidin (AVD), and superoxide dismutase (SOD), streptavidin (SVD), serum amyloid P (SAP), transthyretin (TTR), Salmonella phage P22 tailspike protein (TSP) and the non-SDS-resistant control group (B) triosephosphate isomerase (TIM), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), concanavalin (ConA), β 2-microglobulin (β2M), bovine alpha-lactalbumin (BLA) and yeast alcohol dehydrogenase(ADH). Identical protein samples were either unheated (U) or boiled (B) for 10 min immediately prior to loading onto the gel. Reprinted with permission from (Manning, M., & Colón, W. (2004). Structural basis of protein kinetic stability: Resistance to sodium dodecyl sulfate suggests a central role for rigidity and a bias towards beta sheet structure. *Biochemistry, 43*, 11248-11254). Copyright (2004) American Chemical Society.

KS exhibited by these proteins was confirmed by their native unfolding half-lives, which ranged from 14 min to 19 h (Table 1). The above results support the existence of a correlation between KS and resistance to SDS-induced denaturation. Therefore, SDS-PAGE could serve as a simple method for identifying and selecting KSPs. This method has the advantage that proteins can be easily tested for kinetic stability without having to carry out unfolding experiments. Also, only microgram amounts of sample are needed, and the method is potentially suitable for identifying KSPs present in cell extracts without need for purification. From an application perspective, this assay has the potential of being adaptable for high-throughput applications to enhance the KS of proteins of interest. This could lead to proteins with greater shelf life and/or decreased tendency to aggregate, consistent with the suggestion that the deterioration of an energy barrier between native and pathogenic states as a result of mutation might be a key factor in the misfolding and aggregation of some proteins linked to amyloid diseases (4, 18).

SDS-Resistant			Not SDS-Resistant		
proteins	k_{unf} (s^{-1}) in 0 M GdnHCl	unfolding half-life	proteins	k_{unf} (s^{-1}) in 0 M GdnHCl	unfolding half-life
AVD	$8.1E^{-11}$	270 years	ADH	$8.1E^{-5}$	19 hours
TTR	$9.0E^{-11}$	244 years	TIM	$9.0E^{-5}$	15 hours
PAP	$1.3E^{-10}$	165 years	BLA	$1.3E^{-5}$	12 hours
TSP	$1.6E^{-9}$	13 years	β2M	$1.6E^{-4}$	24 min
SOD	$6.0E^{-9}$	3.7 years	ConA	$6.0E^{-4}$	22 min
CPAP	$8.8E^{-9}$	2.5 years	GAPDH	$8.8E^{-4}$	14 min
SVD	$2.5E^{-8}$	318 days			
SAP	$1.0E^{-7}$	79 days			

Table 1. Unfolding rate constant and half-lives of proteins resistant and not resistant to SDS. Adapted with permission from (Manning, M., & Colón, W. (2004). Structural basis of protein kinetic stability: Resistance to sodium dodecyl sulfate suggests a central role for rigidity and a bias towards beta sheet structure. *Biochemistry, 43*, 11248-11254). Copyright (2004) American Chemical Society.

2. Diagonal two-dimensional (D2D) SDS-PAGE: A proteomics tool for identifying kinetically stable proteins

In the last section, we discussed the correlation between KS and the resistance of proteins to denaturation by SDS, resulting in a simple SDS-PAGE-based assay that is very effective for identifying proteins that have high KS as demonstrated by their resistance to SDS. It is a simple and fast method that could be applied in any lab to test whether a protein is kinetically stable or not. However, the resolution of 1D SDS-PAGE is not sufficient for proteomic research and the KSP bands in a protein mixture are hard to differentiate from non-KSP bands. Therefore, we combined the non-heating and heating SDS-PAGE steps within a single experiment, resulting in a method that we named diagonal two-dimensional (D2D) SDS-PAGE, which combined with mass spectrometry allows the identification of potential KSPs present in complex mixtures. This D2D SDS-PAGE method is similar to previous ones used for the detection of protease susceptibility (Nestler & Doseff, 1997) and to identify stable oligomeric protein complexes in the inner membrane of *E. coli* (Spelbrink, Kolkman, Slijper, Killian, & de Kruijff, 2005). We applied D2D SDS-PAGE to the cell lysate of *E. coli*, and upon proteomics analysis we identified many putative KSPs, thereby giving some insight about potential structural and functional biases in favor and against KS (Xia, et al., 2007).

2.1 Diagonal two-dimensional (D2D) SDS-PAGE method for identifying kinetically stable proteins

In the first step of our D2D SDS-PAGE assay, the unheated sample containing a mixture of proteins is analyzed in the first dimension by SDS-PAGE (Fig. 3A). The gel lane containing the proteins is then cut out and the gel strip is incubated in SDS-PAGE sample buffer and boiled for 10 min (Fig. 3B) before placing above a larger gel for the second dimension run (Fig. 3C). Most proteins will be denatured by SDS even without heating, and thus will migrate the same distance in both gel dimensions, resulting in a diagonal line of spots with a

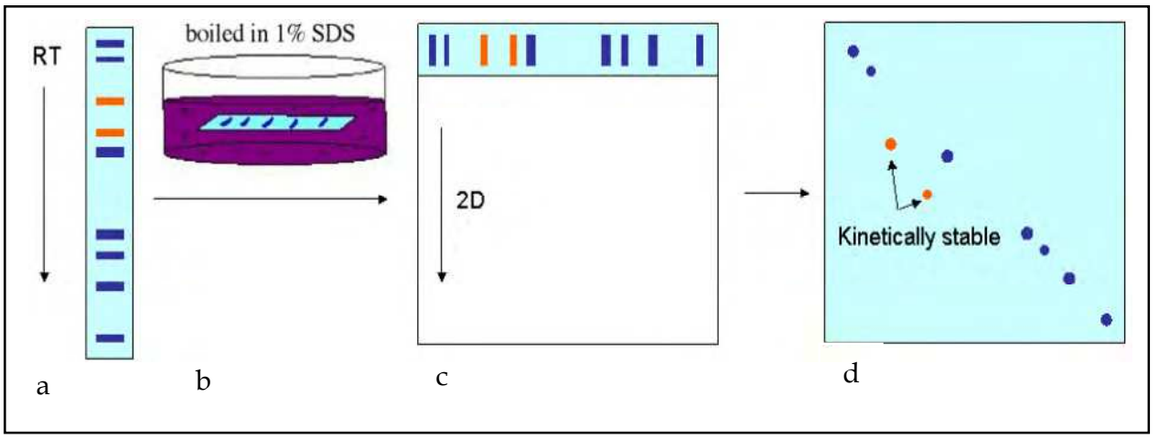


Fig. 3. D2D SDS-PAGE assay for detecting KSPs ((Xia, et al., 2007; Xia, Zhang, Solina, Barquera, & Colon, 2010). After 1D SDS-PAGE, the gel strip is excised and incubated in boiling SDS. The strip is then placed on top of a new gel, followed by a 2nd dimension separation. A diagonal pattern results from the equal migration of non-KSPs in both dimensions. KSPs migrate less in the 1st dimension due to their resistance to SDS, and therefore show up left of the gel diagonal. Reprinted with permission from (Xia, K., Zhang, S., Solina, B. A., Barquera, B., & Colon, W. (2010). Do prokaryotes have more kinetically stable proteins than eukaryotic organisms? *Biochemistry, 49*(34), 7239-7241). Copyright (2010) American Chemical Society.

negative slope across the gel (Fig. 3D). However, SDS-resistant proteins will travel a shorter distance in the first dimension gel and therefore, after the second dimension SDS-PAGE they will end up migrating to a region below the gel diagonal, separated from the bulk proteins. It should be noted that the distance of the spots from the diagonal should not correlate with KS, but rather will depend on several factors, including the oligomeric state, the MW, and the overall charge of the protein.

2.2 D2D SDS-PAGE validation: Identifying the proteome of kinetically stable proteins in *E. coli*

To confirm whether the D2D SDS-PAGE method could detect KSPs from complex mixtures, we applied it to analyze the cell lysate of *E. coli* (Xia, et al., 2007). The D2D SDS-PAGE gel showed the anticipated diagonal pattern arising from the same migration in both dimensions of non-SDS-resistant (i.e. non-kinetically stable) proteins (Fig. 4). Nevertheless, many spots were present below the gel diagonal, and these represent the most abundant KSPs present in the cell lysate of *E. coli*. To identify these proteins, each spot was cut out and subjected to trypsin digestion and proteomics analysis using LC-MS/MS. The resulting MS/MS data were searched against the *E. coli* protein database using the algorithm Mascot 2.1 (Perkins, Pappin, Creasy, & Cottrell, 1999). As reasonable criteria for the correct identification of proteins, we solely included proteins that had at least two peptide hits with a p-value of <0.05, leading to the identification of 50 non-redundant proteins (Table 2). *E. coli* expresses ~884 water-soluble proteins that are observable on a typical 2D gel (Sigdel, Cilliers, Gursahaney, & Crowder, 2004), and therefore, our results indicate that most *E. coli* proteins lack KS. Interestingly, Fig. 4 shows a few unexpected bands and some smearing above the gel diagonal. By adding DTT just

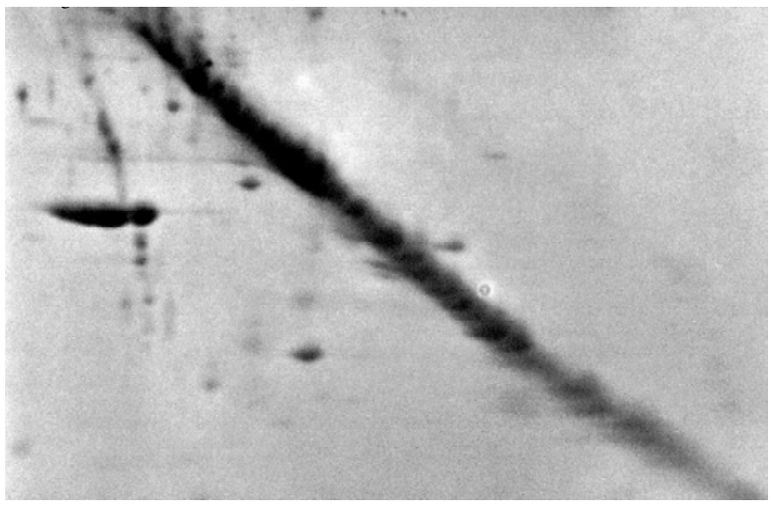


Fig. 4. Analysis of the cellular lysate of *E. coli* by D2D SDS-PAGE (Xia, et al., 2007). The *E. coli* cell lysate was diluted 5-fold and incubated for 5 min in SDS sample buffer (pH 6.8) to a final concentration of 45 mM Tris-HCl, 1% SDS, 10% glycerol, and 0.01% bromophenol blue). A 250 μL aliquot of the lysate solution was loaded without prior heating onto a well of a 12% acrylamide gel (16cm x 14cm x 3mm). The visible spots to the left of the gel diagonal represent the soluble putative KSPs in *E. coli*.

GenBank identifier	**name**	**# . of res**	**2°**	**4°**	**function**
15804817	inorganic pyrophosphatase	176	α/β	6	To catalyze the reaction Diphosphate + H_2O = 2 phosphate
15802070	superoxide dismutase, iron(ii)	193	α/β	2	To destroy toxic radicals which are normally produced within the cells
9507572	chloramphenycol acetyltransferase	219	α/β	3	To covalentely attach an acetyl group from acetyl coA to the chloramphenicol molecule.
443293	triosephosphate isomerase Tim	255	α/β	2	To catalyze the reversible interconversion of triose phosphates isomers dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate.
16131680	uridine phosphorylase	253	α/β	6	To catalyze the reversible phosphorylytic cleavage of uridine and deoxyuridine to uracil and ribose- or deoxyribose-1-phosphate.
14488510	ompf Porin	340	β	3	To form passive diffusion pores to allow low molecular weight hydrophilic materials across the outer membrane
51247607	glycerophosphoryl diester phosphodiesterase	336	α/β	2	To hydrolyze deacylated phospholipids to form glycerol-3-phosphate and the corresponding alcohols
75196280	periplasmic glycerophosphoryl diester phosphodiesterase	371	α/β	2	To hydrolyze deacylated phospholipids to form glycerol-3-phosphate and the corresponding alcohols
11514297	elongation factor, Tu	393	β + α/β	1	To mediate the entry of the aminoacyl tRNA into a free site of the ribosome.

GenBank identifier	name	# . of res	2º	4º	function
15804731	aspartate ammonia-lyase (aspartase)	493	α	4	To catalyze the reversible deamination of the amino acid L-aspartic acid to produce fumaric acid and ammonium ion.
6435772	outer membrane protein Ompx	148	β	3	To neutralize host defense mechanisms
15803823	30S ribosomal protein S4	206	α/β	21	one of the six primary binding proteins to 16s rRNA
16131215	bacterioferritin	158	α	24	iron storage and detoxification
112489962	modulator of drug activity B (MdaB)	204	α/β	2	The MdaB-QuMo operon might protect the cell primarily from more complex quinone compounds.
13786833	pyridoxine 5'-phosphate synthase	242	α/β	8	To catalyze the terminal step in *E. coli* de novo vitamin B6 biosynthesis
2914323	enoyl reductase with bound NAD and benzo-diazaborine	261	α/β	4	To reduce unsaturated acyl carrier protein by reduced pyridine nucleotide
6730179	reduced thioredoxin reductase	320	α/β	2	To reduce thioredoxin
26248038	glyceraldehyde-3-phosphate dehydrogenase	334	α/β	4	Involved in the glycolysis pathway
1421289	S-adenosylmethionine synthetase	383	α/β	2	To catalyze the formation of S-adenosylmethionine
4557950	beta-ketoacyl-acp synthase II	412	α/β	2	To catalyze the condensation reaction of fatty acid synthesis by the addition to an acyl acceptor of two carbons from malonyl-ACP
14278152	2-amino-3-ketobutyrate CoA ligase	401	α/β	2	To catalyze the reaction of Acetyl-CoA + glycine = CoA + 2-amino-3-oxobutanoate.
15804373	transcription termination factor Rho	419	α+β	12	To facilitate transcription termination.
1310928	maltoporin lamb	421	β	3	Involved in the transportation of maltose and maltodextrin
9256952	outer membrane protein Tolc	428	α+β	3	Outer membrane channel and required for proper expression of outer membrane protein genes.
15804852	leucyl aminopeptidase	503	α/β	6	To catalyze the removal of the N-terminal amino acid from most L-peptides
42146	IF2, IF1, and tRNA of E. coli 70S initiation complex	733	α/β	2	Function by binding to the small subunit of the ribosome during the initiation of protein synthesis
91213467	glycerol kinase	537	β	8	To catalyze the formation of glycerol 3-phosphate from ATP and glycerol.
146264	xanthine guanine phosphoribosyltransferase	152	α/β	4	Involved in purine salvage pathway
30065622	purine nucleoside phosphorylase	239	α/β	6	To cleave guanosine or inosine to respective bases and sugar-1- phosphate molecules
223571	protein L12	272	α/β	31	Might be the binding site for factors involved in protein synthesis and be important for accurate translation
75175990	transaldolase	317	α/β	1	To play a role in the balance of metabolites in the pentosephosphate pathway

GenBank identifier	name	# . of res	2°	4°	function
42810	the *E. coli* RNA polymerase alpha subunit amino-terminal domain	329	α+β	2	To catalyze the transcription of DNA into RNA with the four ribonucleoside triphosphates as substrates.
16132149	isoaspartyl dipeptidase	390	α/β	8	To break down β linkages, which are the peptide bonds between the side chain of an aspartate residue and another amino acid
38491472	GroEL	548	α/β	7	A chaperone required for the proper folding of many proteins in prokaryotes, chloroplasts, and mitochondria.
75233972	L-fucose isomerase and related proteins	591	α/β	3	To convert the aldose L-fucose into the corresponding ketose L-fuculose using Mn2+ as a cofactor.
110643069	fructose-bisphosphate aldolase class II	421	α/β	2	To brake down fructose 1,6-bisphosphate into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (DHAP)
15800320	alkyl hydroperoxide reductase, C22 subunit;	187	α/β	10	To reduce organic hydroperoxides in its reduced dithiol form
15804455	glutamine synthetase	469	α/β	12	To catalyze the condensation of glutamate and ammonia to form glutamine.
15799927	phosphoheptose isomerase	192	α/β	4	To catalyze the isomerization of sedoheptulose 7-phosphate in D-glycero-D-manno-heptose 7-phosphate
15804539	catalase; hydroperoxidase HPI(I)	726	α	2	To exhibit both catalase and broad-spectrum peroxidase activities
15799800	dihydrolipoamide dehydrogenase	474	α/β	2	To degrade lipoamide and produce dihydrolipoamide
42377	unnamed protein	549	α+β	2	Unknown
15803853	elongation factor EF-2	704	β + α/β	1	To promote the GTP-dependent translocation of the nascent protein chain from the A site to the P site of the ribosome
15799862	(3R)-hydroxymyristoyl ACP dehydratase	151	α+β	6	Involved in saturated fatty acid biosynthesis
75237743	predicted GTPases	490			Unknown
148247	proline dipeptidase	443			To hydrolyze Xaa-Pro dipeptides and also acts on aminoacylhydroxyproline analogs
15802566	galactitol-1-phosphate dehydrogenase	346			To react with NAD^+ to produce L-Tagatose 6-phosphate And NADH and H^+
38704050	fructose-bisphosphate aldolase	350			Involved in the glycolysis pathway
15801691	putative receptor	353			Unknown
91211384	hypothetical protein UTI89_C2371	374			Unknown

The various columns describe the GenBank identifier number, the name of the protein, the number of residues per subunit, the secondary (2°) structure content, the quaternary (4°) structure content, and the main known function of the protein. This table is adapted from Xia, et al., 2007.

Table 2. Nonredundant subset of SDS-resistant/KSPs proteins in *E. Coli*.

before the heating step, we have confirmed that these bands result from disulfide bond formation during heating. (Xia, et al., 2007)

2.3 Mass spectrometry and identification of KSPs

Protein off-diagonal spots were excised from the gel, washed, reduced, alkylated, and digested in-gel with trypsin overnight. The peptide mixture was extracted, dried and dissolved in 10 μl of 5% formic acid. A Q-TOF 2 mass spectrometer (Waters, Milford, MA) equipped with the CapLC system was used for the LC-MS/MS experiments. We used a trap column of 180 μm ID × 50 mm packed with 10 μm R2 resin (Applied Biosystems, Foster City, CA) connected in series with a 100 μm ID × 160 mm capillary column packed with 5 μm C18 particles. 10μl of the peptide mixture was injected into the trap column at speed of 12 μl/min and desalted for 6 min before being eluted to the capillary column. The peptides were then eluted with final flow rate 250 nl/min by a series of mobile phase B gradients (5 to 10% B in 4 min, 10 to 30% B in 61 min, 30 to 85% B in 5 min, 85 to 85% B in 5 min). Mobile phase A consisted of 0.1% formic acid, 3% acetonitrile and 0.01% TFA, whereas mobile phase B consisted of 0.075% formic acid, 0.0075% TFA in 98/2 acetonitrile/water solution. The mass spectrometer setup was in a data dependent acquisition mode. Ions were selected for MS/MS analysis based on their intensity and charge state +2 to +4. The MS survey scan range is m/z 400-1600 with an acquisition time of 1 sec, whereas the MS/MS fragmentation scan range is m/z 100-2000 with an acquisition time of 2.4 sec. Mascot 2.1 (Matrix Science, London, UK) was used to search all of the MS/MS spectra against the *E. coli* protein database from NCBINR. MS and MS/MS mass tolerance was setup as 1.2 Da and 0.6 Da respectively. PKL files were created by the software Masslynx 3.5 from Waters. The searching parameters setup was as follows: trypsin-specificity restriction with 1 missing cleavage site and variable modifications including oxidation (M), deamidation (NQ), and alkylation (C).

Unlike chemical denaturation, SDS appears to denature proteins by irreversibly trapping them during the transient times in which proteins are unfolded (Manning & Colón, 2004), and since KSPs rarely escape their native state, they are virtually immune to SDS-induced denaturation. Since our initial study (Manning & Colón, 2004) we have analyzed dozens of other proteins and have not observed an exception to this observation. However, there may be other reasons independent of KS that may result in SDS-resistance. For example, proteins that are highly negatively charged may repel SDS. The 50 SDS-resistant *E. coli* proteins we identified in this study have isoelectric points that range from 4-10, and therefore none is expected to electrostatically repel SDS. Also, proteins that are not KS in themselves, but may be part of kinetically stable complexes could lead to false-positives in our assay. A literature search of the proteins listed in Table 1 revealed several proteins that form complexes with GroEL, including S-adenosylmethionine synthase, elongation factor Tu, RNA polymerase α-chain and 50S ribosomal protein L7/L12 (Houry, Frishman, Eckerskorn, Lottspeich, & Hartl, 1999). Interestingly, the GroEL complexes have been shown to be SDS-resistant, whereas GroEL itself and some of its binding partners are known to lack SDS-resistance (Houry, et al., 1999). Thus, D2D SDS-PAGE might also be useful for identifying kinetically stable complexes resulting from the interaction of non-KSP proteins.

2.4 Kinetically stable proteins in *E. coli* have a bias towards enzymatic function

The functions of the KSPs identified by D2D SDS-PAGE were compared with a non-redundant subset of the *E. coli* proteome to determine whether KS is more or less common in proteins with particular functions (Table 2). Interestingly, whereas ~ 32% of all the proteins in *E. coli* are

enzymes, this percentage is ~ 70% in KSPs (Fig. 5A), although there was no preference or aversion for a particular type of enzyme family (Fig. 5B). A larger database will be needed to determine whether this is a general observation. However, it seems plausible that some functions might be more compatible with KS. For example, oxidoreductases might have a predisposition towards KS because they often contain co-factors and metals, and are frequently exposed to potentially harmful free radicals. In contrast, ligase function might require high regulation and flexibility that might be incompatible with KS. (Verdecia, et al., 2003). The absence of kinetically stable transporters and regulators (Fig. 5A) is in agreement with the efficient regulation requirement for these proteins. In particular, KS seems incompatible with transcription factors, which must be quickly turned on and off. Future proteomic analyses of other organisms will increase the number of known KSPs and might provide new insight about the link between protein function and KS.

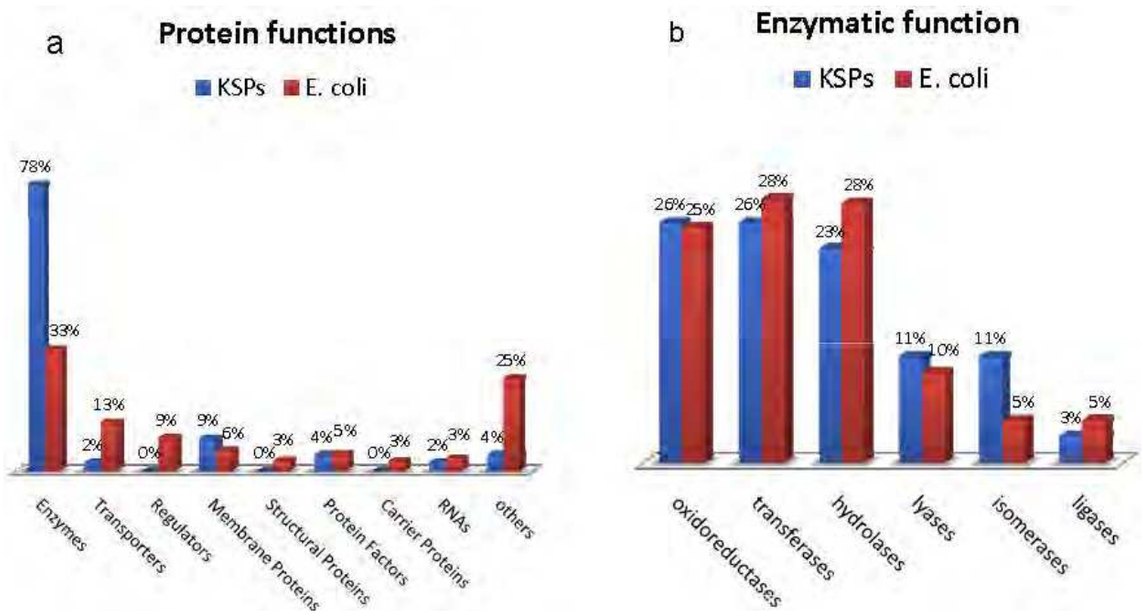


Fig. 5. Different protein (a) and enzymatic (b) functions for a non-redundant subset of the *E. coli* proteome compared to the KSPs identified by D2D SDS-PAGE (Xia, et al., 2007) (a) The kinetically stable subproteome has significantly more enzymes ($p < 0.0001$), but fewer regulators ($p = 0.0082$) and transporters ($p = 0.0076$). Other changes were not statistically significant at the 95% confidence level. Functional assignments were made using the *E. coli* genome and proteome database (GenProtEC). "Other" refers to other functions, including: leader peptides, external origin, cell processes, lipoproteins, pseudogenes, phenotypes, unknown functions, unclassified proteins and sites. (b) Comparison of the six most common enzyme functions does not show statistically significant differences at the 95% confidence level. Enzyme functions were obtained using the BRENDA web site. This figure is adapted from Xia, et al., 2007.

2.5 Monomeric and alpha helical proteins have lower probability of possessing high kinetic stability

Structural analysis of the 50 KSPs identified in *E. coli* (Table 2), yielded 44 that have known 3D structures or are linked to homologs of known structures. To identify potentially structural

features among these KSPs, their secondary (2°) structures were compared to the *E. coli* proteome using the classification obtained by the CATH database (CATH). As shown in Fig. 6A, there was a modest difference in the percentage of β structures compared to the proteins in *E. coli*, but a clear difference in the percentages of α and α/β proteins. Remarkably, very few proteins with all alpha-helical structure were kinetically stable, and none of these were monomeric. Thus it appears that monomeric alpha helical structures might be incompatible with the topological complexity that might be required for KS. Perhaps, α/β proteins might be more likely to possess KS because mixtures of 2° structure lead to more complex topologies.

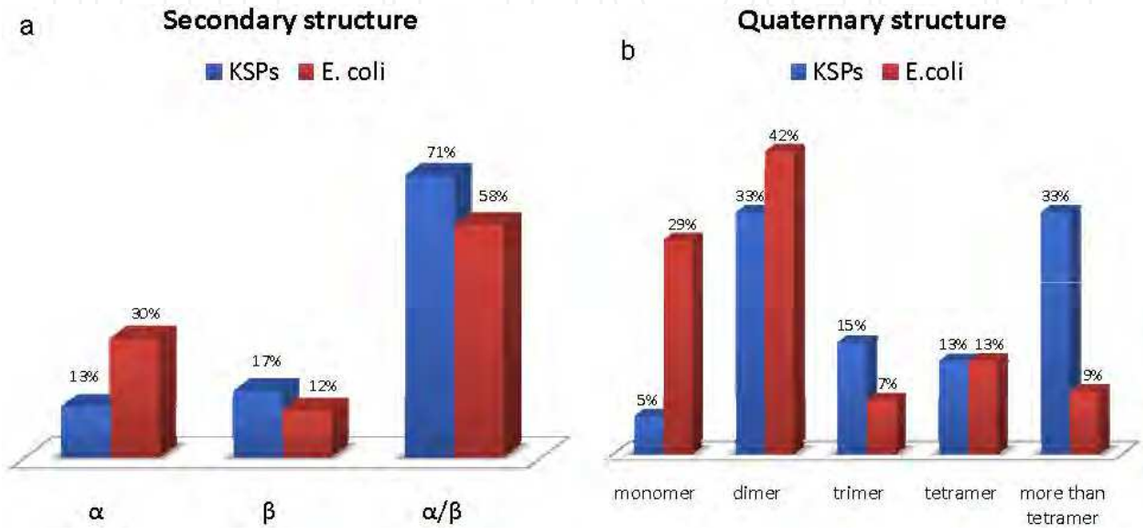


Fig. 6. Secondary (a) and quaternary (b) structure distribution of the non-redundant subset of the *E. coli* proteome compared to the KSPs identified by D2D SDS-PAGE. (a) The KSPs have fewer (p=0.0034) all alpha-helical proteins compared to the rest of the *E. coli* proteome. Structure classifications were made using the CATH database (CATH). (b) The KSPs include only a few monomers (p=0.0002), and significantly more large oligomeric structures with at least five subunits (p<0.001). Dimers and tetramers occur at approximately the same frequencies. Quaternary structure information was obtained from the PQS Protein Quaternary Structure database ("EMBL-EBI PQS Protein Quaternary Structure database "). This figure is adapted from Xia, et al., 2007.

Analysis of the oligomeric/quaternary (4°) structures show that the percentage of monomeric KSPs in *E.coli* is much lower compared to the whole proteome, whereas the percentage of oligomeric proteins with 5 or more subunits is significantly higher (Fig. 6B). Although it is not clear why higher oligomeric structures might favor KS, they might confer greater rigidity and protection of surface residues from water.

2.6 Thermophilic and mesophilic prokaryotes have a greater number of kinetically stable proteins than eukaryotic organisms

D2D SDS-PAGE provides a unique opportunity to investigate the proteome of KSPs in diverse organisms. Therefore, we studied the cell lysates of the thermophilic bacteria *Thermus thermophilus* and *Thermus aquaticus*, and the archaea *Sulfolobus acidocaldarius*, which grow at optimal temperatures of 65, 70, and 80°C, respectively (Xia, Zhang, Solina, Barquera, & Colon, 2010) These thermophiles exhibited high number of SDS-resistant (i.e.

KSPs) proteins (Fig. 7A). In contrast, the mesophilic bacteria *Escherichia coli* (Xia, et al., 2007), *Vibrio cholerae*, and *Bacillus subtilis*, showed significant variation and fewer KSPs than the thermophiles, especially in the upper left area of the gel where the higher molecular weight proteins migrate (Fig. 7B). We also studied three very different eukaryotic organisms from separate kingdoms, including *Saccharomyces cerevisiae*, maize, and *Tetrahymena thermophila*. Remarkably, these eukaryotic organisms exhibited very few, if any, KSPs (Fig. 7C). Therefore, our results clearly showed that thermophiles and prokaryotes have more KSPs than mesophiles and eukaryotes, respectively(Xia, et al., 2010)

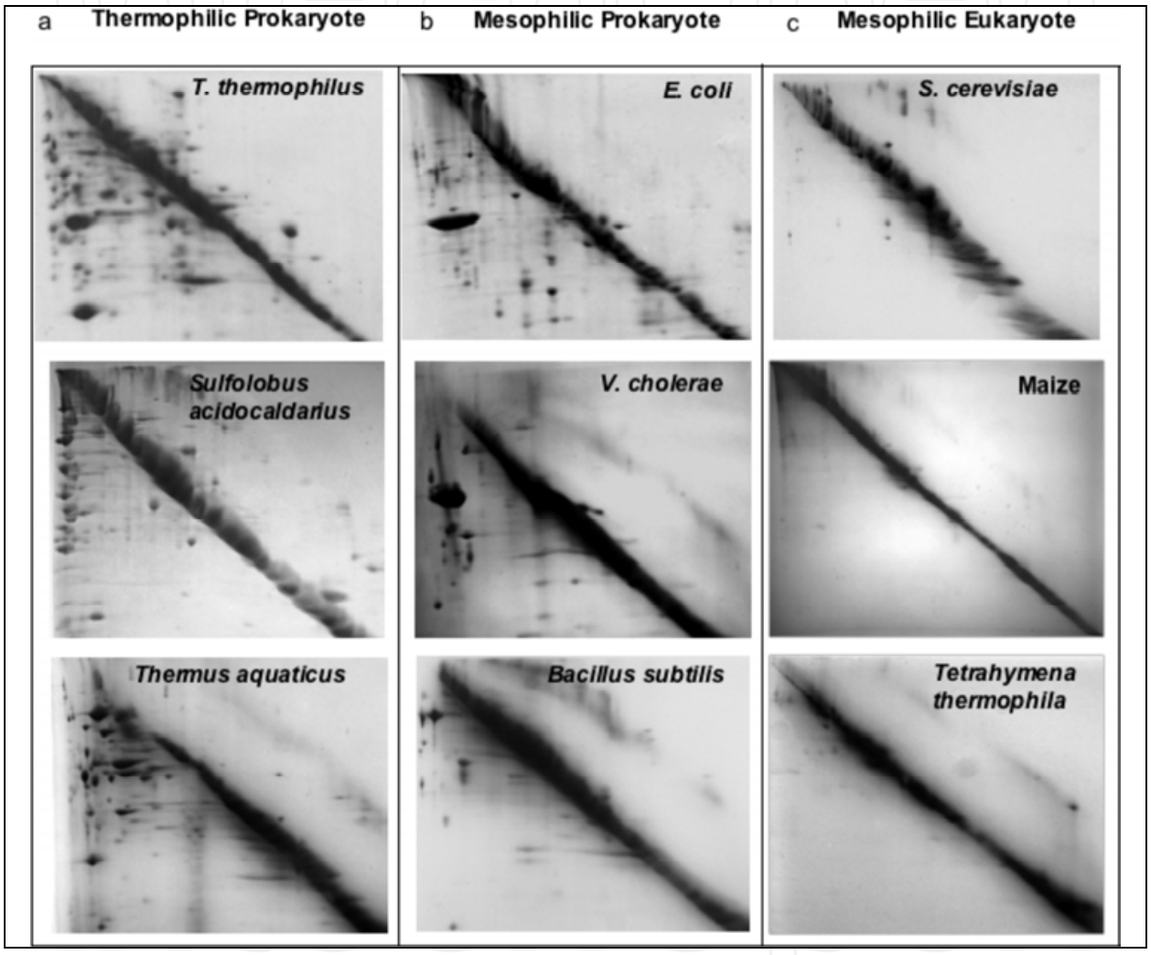


Fig. 7. D2D SDS-PAGE of the lysate of various organisms to probe the extent of kinetically stable proteins present (Xia, Zhang, Solina, Barquera, & Colon, 2010). (a) Thermophilic prokaryotes (*T. thermophilus*, *S. acidoldarius*, and *T. aquaticus*) exhibited significantly more spots migrating to the left of the diagonal than (b) mesophilic prokaryotes (*E. coli*, *V. cholerae*, and *B. subtilis*). (c) Mesophilic eukaryotes (*Sa. cerevisiae*, maize, and *Te. thermophila*) showed the fewest number of KSPs spots. Because of differences in background staining, the pictures were slightly enhanced by Microsoft office picture manager through linear adjustment of contrast, brightness, and color applied to the entire image in each case. Reprinted with permission from (Xia, K., Zhang, S., Solina, B. A., Barquera, B., & Colon, W. (2010). Do prokaryotes have more kinetically stable proteins than eukaryotic organisms? *Biochemistry, 49*(34), 7239-7241). Copyright (2010) American Chemical Society.

The results of this study suggest that KS might be a very significant feature of certain proteins required for the adaptation and survival of microbial organisms, which lack cellular sub-compartments, and possess a primordial defense system. In contrast, eukaryotes might be less dependent on KS for survival, and this property might not be generally compatible with the regulatory demands of these more sophisticated organisms. Thus, the presence of many KSPs in prokaryotes, especially thermophiles, suggests that this property is essential for the survival of these simpler organisms. Proteomics analysis of KSPs in thermophilic organisms might reveal a subset of critical proteins that play a major role in determining the ability of these organisms to live and thrive at higher temperatures.

3. Proteomics analysis of KSPs by capillary electrophoresis

3.1 Capillary electrophoresis as an effective method to detect KSPs

Based on the previous correlation between KS and a protein's SDS-resistance, we set out to explored whether SDS-capillary electrophoresis (CE) would be suitable for identifying KSPs (Zhang, Xia, Chung, Cramer, & Colon, 2010). We used eight control proteins, including four KSPs and four non-KSPs. The unheated samples of the non-KSPs α-chymotrypsin (CHT), glucose dehydrogenase (GD), concanavalin A (ConA) and myoglobin (MYO) were denatured by SDS, resulting in identical migration on the gel as the respective samples that were boiled. In contrast, the unheated samples of the KSPs glucose oxidase (GO), streptavidin (SVD), superoxide dismutase (SOD), and subtilisin carlsberg (SCA) were resistant to SDS and exhibited a slower migration on the gel. Analysis of these proteins by CE, which is based on the same electrophoretic principles as SDS-PAGE, showed results consistent with SDS-PAGE (Fig. 8). The CE data for the 4 non-KSPs showed that all boiled

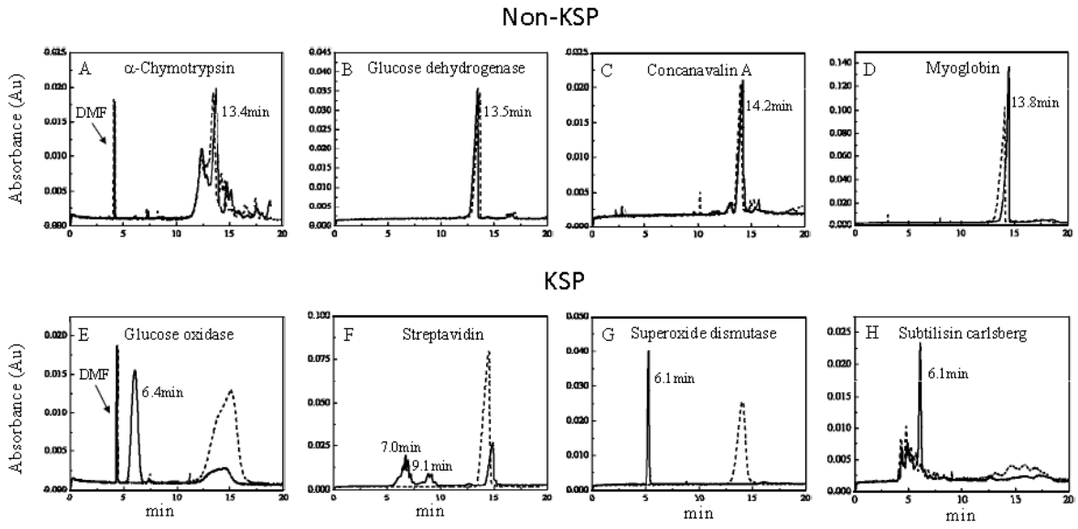


Fig. 8. Electropherograms showing the migration of unboiled and boiled samples of non-KSPs (A–D) and KSPs (E–H). Black solid lines and dash lines represent the data of samples incubated in SDS that were not boiled or boiled, respectively. Samples were incubated in 20 mM sodium phosphate buffer (pH 7.4) containing 1% (w/v) SDS for 10 min. The electropherograms of unboiled and boiled non-KSPs showed little difference, but unboiled KSPs had significantly faster migration. This figure is adapted from Zhang, Xia, Chung, Cramer, & Colon, 2010.

and unboiled non-KSPs had similar migration times of 13.4 - 14.2 min (Fig. 8A-D). Since KSPs bind few SDS molecules, the KSPs had faster CE migration time of 6-7 min. (Figure 8E-H). In most cases there were also one or more smaller peaks in the 14-15 min region, similar to that of non- KSP, suggesting that the protein was partially denatured by SDS. Interestingly, the broader and/or multiple peaks observed in Figs. 8F and H suggest conformational heterogeneity and might arise by the presence of different population of species.

We used a fused silica capillary to separate the proteins, and therefore, the positively charged cations of the buffer solution interact with the negatively charged silanoate groups and form a mobile cation layer. Under normal polarity with the anode (+) at the sample inlet and the cathode (-) at the sample outlet, the mobile cation layer is pulled in the direction of the negatively charged cathode. The solvation of these cations cause the bulk buffer solution to migrate with the mobile layer, producing the electro-osmotic flow (EOF). Thus, the protein:SDS complexes of denatured proteins are highly negatively charged and experience more repulsion from the cathode (outlet), resulting in slower migration than the relatively SDS-free KSPs.

3.2 Using capillary electrophoresis to identify the proteome of KSPs

In CE, proteins are typically detected using UV absorption, laser induced fluorescence (LIF), or by coupling to a mass spectrometer (MS) (Fonslow & Yates, 2009; Garcia-Campana, Taverna, & Fabre, 2007; Herrero, Ibanez, & Cifuentes, 2008; Kasicka, 2008; Stutz, 2005). A limitation when using UV detection is that the limit of detection (LOD) is in the micromolar range. However, this disadvantage can be overcome by using other detectors. The sensitivity of LIF is subnanomolar (Gutman & Kessler, 2006) and a MS detector could provide amol-range sensitivity (Gaspar, Englmann, Fekete, Harir, & Schmitt-Kopplin, 2008; Haselberg, de Jong, & Somsen, 2007; Hernandez-Borges, Borges-Miquel, Rodriguez-Delgado, & Cifuentes, 2007; Tempels, Underberg, Somsen, & de Jong, 2007). Thus, it is possible to interface a CE instrument with a LTQ orbitrap MS using an Agilent sheath-flow adapter kit that can be used with any ESI-MS instrument. The method of choice for coupling CE to ESI/MS is the coaxial sheath-flow interfacing, which is stable, and provides the best sensitivity (in the amol range). Stability and sensitivity of this interfacing has been confirmed by a number of studies (Gaspar, et al., 2008; Haselberg, et al., 2007; Hernandez-Borges, et al., 2007; Tempels, et al., 2007).

We have shown that under certain conditions (Zhang, et al., 2010), KSPs will move faster in CE than non-KSPs. Since most proteins in any organism are not kinetically stable, they will migrate slower and together due to their similar z/m value. In contrast, the KSPs will have a lower and variable z/m value that will allow CE to separate with high resolution the low abundant KSPs from the bulk of the non-KSPs. A description of the general approach of the proposed CE experiment is shown in Fig. 9. In terms of the CE instrument, the SDS concentration, the voltage, the capillary length, and the loading amount should be optimized to achieve the best separation.

3.3 Limitations of D2D SDS-PAGE and advantages of CE

Although 2D electrophoresis (2DE) is often used to separate proteins for proteomics analysis, it has several disadvantages. Since MS analysis is limited to proteins that can be visualized, only abundant proteins can be seen. 2DE is also time-consuming and does not lend itself

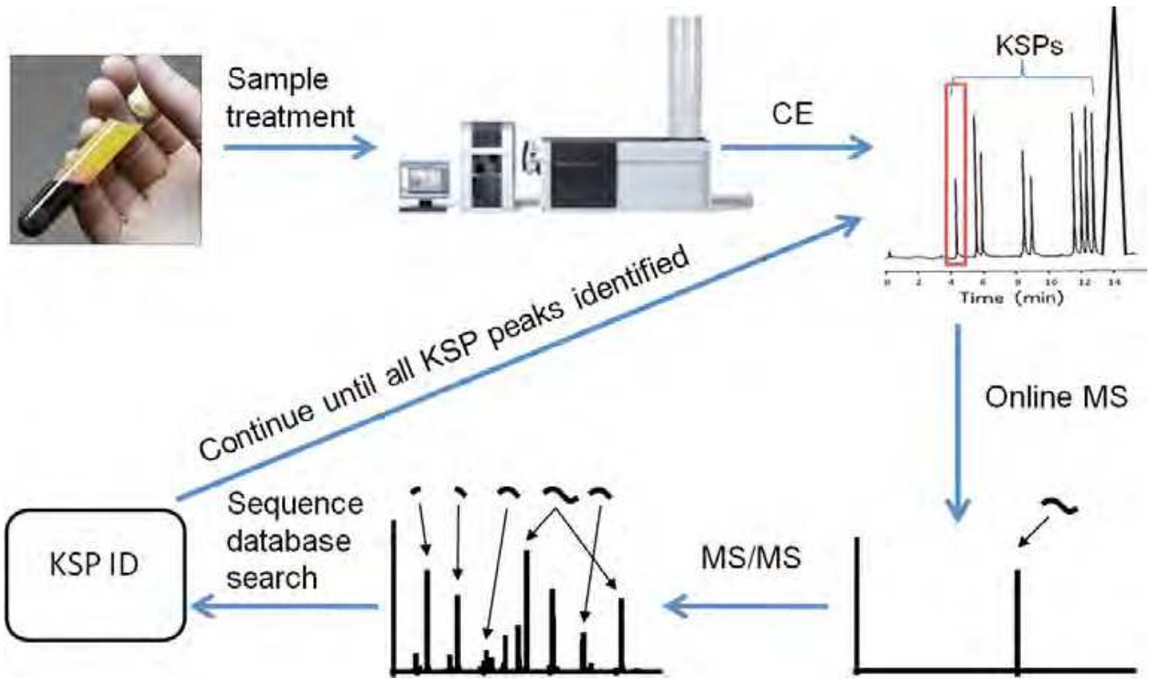


Fig. 9. Identifying the KSPs of an organism by CE-MS. KSPs will be separated from non-KSPs by CE and eluted directly into an online orbitrap mass spectrometer. Following measurement of its mass, intact KSP are directly fragmented in the machine. The mass of its daughter fragments is measured and then analyzed using a database to determine the identity of the KSPs.

to automation. In addition, each step requires lengthy optimization and user intervention. Furthermore, sample handling can easily introduce protein loss and artifacts, such as oxidation and other side-chain modifications. Since CE is based on the same general electrophoretic principle as PAGE, we were able to show that CE can also be used to identify KSPs (Fig. 8) (Zhang, et al.,2010). CE is an efficient and highly sensitive separation method that is widely used in biochemical and pharmaceutical research (Kostal, Katzenmeyer, & Arriaga, 2008; Little, Paquette, & Roos, 2006; McEvoy, Marsh, Altria, Donegan, & Power, 2008). CE is fast and cost-effective, and allows high sample throughput, easy automation, separation efficiency, precision, and only requires nanoliter volumes of sample (Dolnik, 2006, 2008). The small diameter of the capillaries allows better heat dissipation than gel electrophoresis, thereby minimizing band broadening. CE, especially when using an MS detector, could analyze very small amounts of sample with high sensitivity. For example, MS can identify a 1 pmol spot on a gel, whereas in the case of CE, it is possible to identify a 1 fmol amount of protein, and perhaps up to 1 amol with an orbitrap MS instrument (Dolnik, 2006, 2008). Table 2 provides a side-by-side comparison between D2D SDS-PAGE and CE. Thus, although D2D SDS-PAGE is accessible and affordable for identifying KSPs in complex organisms, CE-MS is more promising for faster analysis and for identifying KSPs that have low abundance. Furthermore, the sharpness of the peaks observed by CE might reveal valuable and unique information about the conformational heterogeneity of KSPs and the extent of protein KS.

Comparison	D2D-SDS-PAGE	Capillary Electrophoresis
Instrument cost	$2k	≥ $70k
Time cost (one sample)	more than 10h plus digestion and MS time	less than 1h
Technical demands	laborious, need experience and good hands	automated procedure
Reproducibility of result	could be variable depending on technical expertise	reproducible
Sample amount	0.3~1mg protein	nl~ml (1ng-1mg) protein
Resolution	10,000 for 2DE, up to one hundred KSP spots could be separated in a single gel.	>1000 peptides
Sensitivity*	coomassie stain – pmol florescence stain - fmol	MS detector - fmol
MS coupling	N/A	online connection
Extra information	spot pattern	retention time

*A 1 pmol coomassie stain can be identified by MS, but a fmol fluorescence spot cannot due to protein loss (e.g. crosslinking to the gel, getting trapped by the surface of pipette tip or tube) during the in-gel digestion process.

Table 3. Comparison of D2D-SDS-PAGE and CE

4. Conclusion

The D2D SDS-PAGE method described here is simple and accessible for the proteomics-level identification of KSPs. In contrast the SDS-CE methods is fast, sensitive, and has the potential to be applied in high throughput fashion. The key feature of both methods is their ability to separate SDS-resistant (i.e. KSPs) from non-SDS-resistant (i.e. non-KSPs) proteins. Therefore, mild conditions must be employed during the separation step to preserve the conformational integrity of the proteins. Afterwards, conventional proteomics analysis may be carried out to identify KSPs.

Living organisms have a diversity of sub-proteomes that are involved in different pathways or functions. The sub-proteome of KSPs is likely to include proteins that must have longer half-lives and possess resistance to degradation for the benefit of the organism. The SDS-based methods we have developed will make it possible to study a variety of systems, including the cellular lysates of microorganisms, human plasma and other biological fluids, normal and diseased cells, and all types of plants and food materials. Such studies will increase the database of KSPs and will facilitate investigation of the structural basis and the diverse functional roles of KS. Furthermore, they will stimulate research to understand the biological and pathological roles of the abnormal gain or loss of KS in proteins.

5. Acknowledgment

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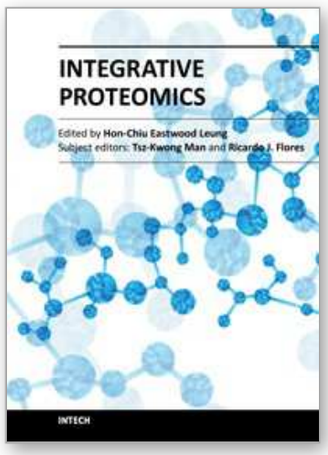
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Proteomics was thought to be a natural extension after the field of genomics has deposited significant amount of data. However, simply taking a straight verbatim approach to catalog all proteins in all tissues of different organisms is not viable. Researchers may need to focus on the perspectives of proteomics that are essential to the functional outcome of the cells. In Integrative Proteomics, expert researchers contribute both historical perspectives, new developments in sample preparation, gel-based and non-gel-based protein separation and identification using mass spectrometry. Substantial chapters are describing studies of the sub-proteomes such as phosphoproteome or glycoproteomes which are directly related to functional outcomes of the cells. Structural proteomics related to pharmaceutics development is also a perspective of the essence. Bioinformatics tools that can mine proteomics data and lead to pathway analyses become an integral part of proteomics. Integrative proteomics covers both look-backs and look-outs of proteomics. It is an ideal reference for students, new researchers, and experienced scientists who want to get an overview or insights into new development of the proteomics field.

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