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Reproducible enrichment of extracellular heat shock proteins from blood serum using monomeric avidin

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ABSTRACT

Extracellular heat shock proteins (eHsps) in blood circulation have been associated with various diseases, including cancer. However, the lack of methods to enrich eHsps from serum samples has hampered the characterization of eHsps. This Letter presents our serendipitous finding that the monomeric avidin resin can serve as an affinity resin to enrich eHsps from blood serum. Biochemical mechanism of this eHsp enrichment as well as implications in biomarker discovery is discussed.

Heat shock proteins (Hsps) are widely known as cytosolic proteins involved in the maintenance of protein folding.[1,2] Maintenance of native protein structures is important because denaturation of proteins can trigger aggregations, which are harmful to the cell. Hsps bind to the hydrophobic surfaces that are exposed on partially denatured proteins. The binding of Hsps prevents aggregations, thereby enabling proteins to refold to their native structures.[3,4] Although Hsps have long been considered as cytosolic proteins, some of them were recently found in the extracellular milieus, such as tumor surface and blood circulation.[5–7] These extracellular Hsps (eHsps) appear to be associated with various diseases including cancer and cardiovascular diseases.[8,9] However, minuscule quantities of eHsps in blood circulation make it difficult to characterize their abundance and distribution in a reproducible manner.

In addition to the minuscule quantities, characterization of eHsps is complicated by several other factors. First, Hsp proteins have a large number of variants because of mRNA isoforms and post-translational modifications.[10,11] At present, it is not clear which variants exist in the extracellular milieus. As such, antibody-based methods, such as enzyme-linked immunosorbent assay (ELISA), might not provide comprehensive profiles of eHsps. Second, direct analysis of eHsps by mass spectrometry (MS) is difficult because the predominant serum proteins, such as albumin and immunoglobulins, would obscure the signals of minor constituents.[12,13] Although there are commercial kits to deplete ~90% of albumin and immunoglobulin in serum, reproducible detection of low abundance proteins is still difficult because of the large dynamic range of serum protein concentration (10 orders of magnitude or higher).[13] Ideally, what is needed is a method to selectively enrich eHsps from serum samples.

Here, we report a simple approach to reproducibly enrich Hsps from complex proteomes, such as cell lysates and serum samples. This approach is based on our serendipitous finding that monomeric avidin resin binds selectively to a variety of Hsps in cell lysates. Monomeric avidin is an affinity resin in which stereptavidin monomers are immobilized on agarose beads. It is prepared by partial denaturation of immobilized tetrameric (native) stereptavidin.[14] Monomeric and tetrameric avidin resins are widely used for affinity purification of biotinylated macromolecules.[15] The tetrameric avidin resin has high affinity and specificity to biotin (K_d = ~10^{-15} M),[15] whereas monomeric avidin has a reduced affinity (K_d = 10^{-7} M). The reduced affinity of monomeric avidin is useful in applications that require the capture and release of biotinylated proteins under mild conditions.[14,16] Our recent studies, however, revealed that monomeric avidin also captures non-biotinylated proteins in cell lysates. MS-based profiling showed that a series of Hsps are the major components of the enriched proteins. Further studies demonstrated that eHsps could be enriched from serum without the contamination of abundant serum proteins.

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The ability of monomeric avidin to enrich Hsps was discovered during a study on photochemically biotinylated proteins in cell lysates. During this study, background protein-binding to monomeric avidin was assessed using the cytosolic lysate of Jurkat cells. The Jurkat lysate was incubated with monomeric avidin and then washed with Tris-buffered saline (TBS) containing 0.1% Tween 20 to remove non-specifically bound proteins. SDS–PAGE of the bound proteins, followed by Coomassie blue staining, revealed many bands distinctly different from the profile of Jurkat cytosolic lysate (Fig. 1, lanes 1 and 2). On the other hand, when the background binding to tetrameric avidin was examined, no visible bands were observed (Fig. 1, lane 3). The absence of background binding to tetrameric avidin suggested that the proteins captured by monomeric avidin were not endogenously biotinylated proteins. Clearly, some other mechanism of affinity purification was at play.

In order to gain insights into this mysterious affinity purification, we conducted mass spectrometric analysis of proteins enriched by monomeric avidin. Trypsin digestion of the captured proteins, followed by MS/MS analysis, revealed a profile of nearly 60 proteins with the Mascot score of 50 or higher (see Table S1 in the Supplementary data for the complete list of proteins enriched by monomeric avidin). While the list contained abundant cytosolic proteins, such as tubulins and actins, a series of Hsps stood out as the most distinct family of proteins, which included four chaperonin containing TCP1 isoforms (Hsp60s), six Hsp70 isoforms, and four Hsp90 isoforms (Table 1). The current study found more Hsps in Jurkat cytosol than a previous proteomic study,[17] in which Jurkat lysates, including cytosol fraction, were subjected to mass spectrometry-based profiling. Thus, our finding suggests the importance of the enrichment process to obtain comprehensive profiles of Hsps.

The observed enrichment of Hsps by monomeric avidin can be rationalized when we consider the structural difference between monomeric and tetrameric avidin resins. As mentioned earlier, monomeric avidin resin is prepared from partial denaturation of tetrameric avidin.[14] In this procedure, immobilized tetrameric avidin is subjected to a chemical denaturant, such as urea or guanidine hydrochloride, which causes the dissociation of avidin tetramers into individual monomers. Non-immobilized subunits are removed from the resin at this stage (Fig. 2c). The remaining avidin proteins are then renatured to generate the monomeric avidin matrix. The resulting monomers are still partially denatured because they cannot reform the native tetrameric structure.[14] Due to the partial denaturation, immobilized avidin monomers expose the internal hydrophobic core that can be recognized by Hsps (Fig. 2). Thus, the production of monomeric avidin inadvertently creates affinity-resin for Hsps. Tetrameric avidin resin, on the other hand, has hydrophobic surfaces buried inside, which explains the absence of background protein binding.

The observed enrichment of Hsps by monomeric avidin opened a possibility that the resin could be used to enrich eHsps from serum. To test this, human serum samples from donors with and without colorectal cancer were incubated with monomeric avidin resin. This time, samples were washed under stringent conditions (ten times with TBS containing 2% Tween 20) to prevent non-specific binding of abundant serum proteins, such as albumin. Tightly bound proteins were denatured and separated by SDS–PAGE. Proteins were then visualized with Coomassie blue and silver stains (Fig. 3a and b, respectively). The Coomassie stained gel indicated

Table 1
Heat shock proteins enriched from Jurkat cytosolic lysate using monomeric avidin affinity resin

GI[a]	Mass	Gene
83699649	98,652	Heat shock 90 kDa protein 1, alpha
15010550	90,309	Heat shock protein gp96 precursor
306891	83,584	90 kDa heat shock protein
67477458	80,345	Heat shock protein 75 kDa, mitochondrial precursor
62089222	78,018	Heat shock 70 kDa protein 1A variant
3522	71,209	Heat shock 70 kDa protein 6 (HSP70B')
5729877	71,082	Heat shock 70 kDa protein 8 isoform 1
21759781	70,748	Heat shock 70 kDa protein 1-like
188488	70,294	Heat shock-induced protein
4204880	70,237	Heat shock protein
5453607	59,842	Chaperonin containing TCP1, subunit 7 isoform a
1136741	59,035	Chaperonin containing TCP1, subunit 8 (theta)
4502643	58,444	Chaperonin containing TCP1, subunit 6A isoform a
1200184	58,316	Chaperonin containing TCP1, subunit 4 (delta)

[a] GI: Gene identifier.

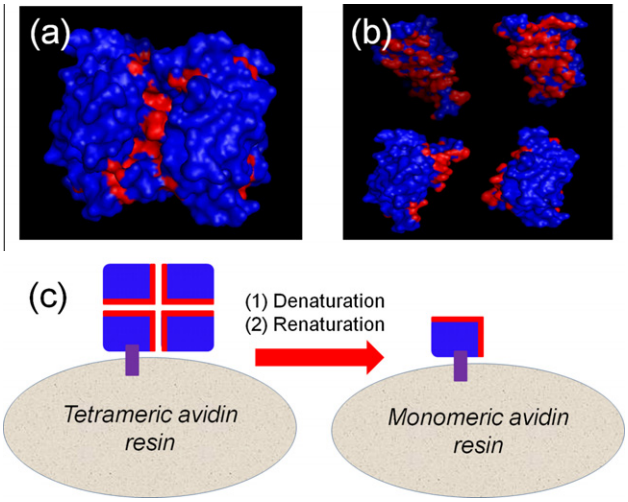

Figure 2. Breakage of tetrameric avidin results in the exposure of interior surfaces. (a) Tetrameric streptavidin (PDB 3MG5). Avidin–avidin interfaces, which are mostly composed of hydrophobic residues, are shown in red, whereas the external hydrophilic surfaces are shown in blue. (b) Monomeric avidin exposes the internal hydrophobic surfaces which serve as the bait for Hsps. (c) Schematic diagram of monomeric avidin resin preparation, in which immobilized tetrameric avidin is chemically denatured and then renatured. A covalent bond between an avidin protein and resin is shown as a purple line. Only covalently attached avidin protein remains on the resin after renaturation.

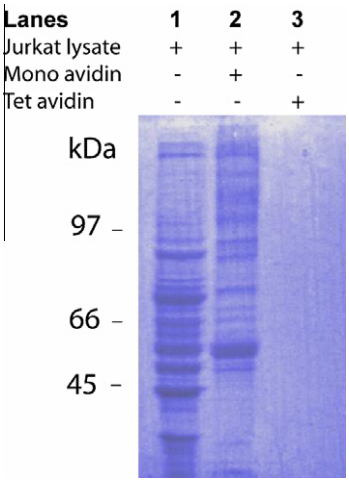

Figure 1. Selective protein enrichment by monomeric avidin. Jurkat cytosolic lysate was incubated with one of the avidin affinity resins. After washing, bound proteins were separated by SDS–PAGE and visualized with Coomassie blue stain. Lane 1: Jurkat cytosolic lysate (no avidin resin treatment); lane 2: Proteins that bound to monomeric avidin resin; lane 3: Proteins that bound to tetrameric avidin resin (no visible bands were observed).

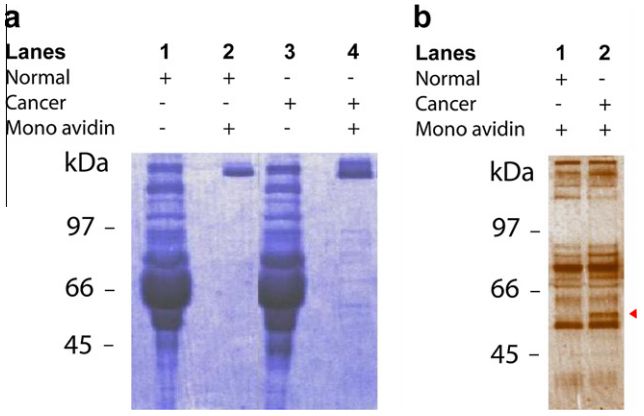

Figure 3. Affinity purification of serum proteins using monomeric avidin resin. Serum samples from a healthy donor (normal) and a donor with colon cancer (cancer) were incubated with monomeric avidin resin. Proteins bound to the resin were separated by SDS–PAGE and visualized by (a) Coomassie-blue staining and (b) silver staining. A notable difference between the two was a silver-stained band around 55–60 kDa (red arrow head).

that our stringent washing procedure eliminated abundant proteins, which predominated the original serum samples (Fig. 3a, lanes 1 and 3). Although the enriched proteins were barely visible on Coomassie stained gel,[18] the silver staining showed distinct bands for each sample. At this stage, however, it was not clear whether the enriched proteins included eHsps.

Since the silver stained gel showed a notable difference around 55–60 kDa (Fig. 3b, red arrow), the two samples were subjected to Western blot analysis using an anti-human Hsp60 antibody. Western blot revealed that the enriched proteins indeed included eHsp60 (Fig. 4a). Reproducibility of the method was also examined by a replicate analysis, in which four independent replicates of each sample were examined. The analysis found the relative standard deviation of 20% or lower, which allowed the detection of a subtle difference in eHsp60 between the two samples (p <0.05). Although further studies are needed to determine whether the observed difference is due to colon cancer, the current study demonstrated the utility of monomeric avidin as a new affinity resin for eHsps.

Although serum is the ideal source of biomarkers, the interference by abundant proteins has been a major technical hurdle that has hampered the discovery of new serum biomarkers.[12,13] This is an important problem because there is a clear and urgent need for new methods for early detection of cancer and other serious diseases. Most existing serum biomarkers suffer from poor specificity and sensitivity. For example, cancer antigen 125 (CA125) detects only 50% of the early stage ovarian cancer.[19] The prostate-specific antigen (PSA) test cannot differentiate the various stages of prostate cancer.[19] Although the advent of powerful MS tools has shown a great promise in biomarker discovery,[20] the interference by the predominant serum proteins continues to be a major problem that circumscribes our ability to explore new serum biomarkers.

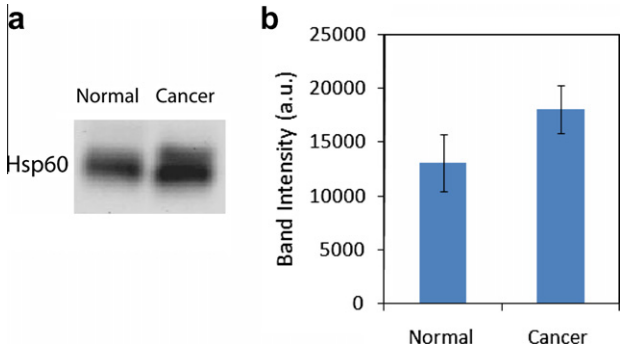

Figure 4. Expression levels of Hsp60 in serum samples using anti-human Hsp60 antibody. (a) Western blot of serum samples. (b) Quantification of the labeling signals for normal and cancer serum samples. Results are the average values ± standard deviations for four independent experiments (p <0.05).

Enrichment of low-abundance proteins is becoming an increasingly important concept in biomarker discovery. Several new technologies have been developed for the enrichment and detection of low-abundance proteins. For example, one approach uses peptide library beads, in which immobilized peptide ligands are used to enrich their target proteins.[21] In a similar approach peptoid library beads have been employed to enrich proteins in complex proteomes.[22] Furthermore, immobilized carbohydrate antigens have been used to enrich specific populations of antibodies from blood serum.[23]

What separates our method from others, however, is the use of partially denatured proteins for the purpose of proteomic analysis. Immobilization of multimeric proteins, followed by denaturation and renaturation, creates molecular baits to enrich proteins that recognize denatured or misfolded motifs. In addition to the enrichment of Hsps, such affinity resins can be employed to gain new insights into various diseases associated with protein misfolding, such as neurodegenerative diseases, cystic fibrosis, and some types of cancer.[24,25]

In conclusion, our current study revealed the ability of monomeric avidin to reproducibly enrich eHsps from serum samples. The method opens a new opportunity to characterize the distribution and abundance of eHsps in the context of various human diseases.

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Supplementary data

Supplementary data (the complete profile of Jurkat cytosolic proteins enriched with monomeric avidin and experimental procedure) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.05.111.

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