Journal of Immunological Methods 428 (2016) 9–19

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Isolation of soluble scFv antibody fragments specific for small biomarker molecule, L-Carnitine, using phage display

Rabab M. Abou El-Magd [a,f], Nicolas F. Vozza [a], Jack A. Tuszynski [c,d,e,*], David S. Wishart [a,b]

[a] Department of Biological Sciences, University of Alberta, CCIS 5-012, Edmonton, AB T6G 2E1, Canada
[b] Department of Computing Sciences, University of Alberta, Edmonton, AB T6G 2E8, Canada
[c] Experimental Oncology, Department of Oncology, University of Alberta, Canada
[d] Cross Cancer Institute, 11560 University Avenue, Edmonton, AB T6G 1Z2, Canada
[e] Department of Physics, University of Alberta, Edmonton, AB, Canada
[f] Genetic Engineering and Biotechnology Research Institute, City of Scientific Research and Technological Applications, 21934 New-Burgelarab City, Alexandria, Egypt

ARTICLE INFO

Article history:
Received 12 September 2015
Received in revised form 23 October 2015
Accepted 18 November 2015
Available online 1 December 2015

Keywords:
L-Carnitine
Single chain antibody fragment (scFv)
Phage display
Enzyme-linked immunosorbent assay (ELISA)
Fluorescence quenching assay
Circular Dichroism (CD)

ABSTRACT

Isolation of single chain antibody fragment (scFv) clones from naïve Tomlinson I + J phage display libraries that specifically bind a small biomarker molecule, L-Carnitine, was performed using iterative affinity selection procedures. L-Carnitine has been described as a conditionally essential nutrient for humans. Abnormally high concentrations of L-Carnitine in urine are related to many health disorders including diabetes mellitus type 2 and lung cancer. ELISA-based affinity characterization results indicate that selectants preferentially bind to L-Carnitine in the presence of key bioselecting component materials and closely related L-Carnitine derivatives. In addition, the affinity results were confirmed using biophysical fluorescence quenching for tyrosine residues in the V segment. Small-scale production of the soluble fragment yielded 1.3 mg/L using immunopure-immobilized protein A affinity column. Circular Dichroism data revealed that the antibody fragment (Ab) represents a folded protein that mainly consists of β-sheets. These novel antibody fragments may find utility as molecular affinity interface receptors in various electrochemical biosensor platforms to provide specific L-Carnitine binding capability with potential applications in metabolomic devices for companion diagnostics and personalized medicine applications. It may also be used in any other biomedical application where detection of the L-Carnitine level is important.

© 2015 Elsevier B.V. All rights reserved.

1. Introduction

L-Carnitine (HMDB00062) is a quaternary amine that occurs naturally in most mammalian tissues. It is not an essential amino acid; as it can be synthesized in the body; but it has been described as a conditionally essential nutrient for humans, which is considered to be a valuable small biomolecule.

L-Carnitine facilitates entry of long-chain fatty acids into mitochondria for utilization as fuel and facilitates removal from mitochondria of short-chain and medium-chain fatty acids that accumulate as a result of normal and abnormal metabolism (Liu et al. 2004).

L-Carnitine and its acyl esters may act as antioxidants either by having a primary anti-oxidant activity through inhibiting free radical generation, scavenging the initiating free radicals, or terminating the radical propagation reactions. More likely, however, it may function as a secondary antioxidant through repairing oxidized polyunsaturated fatty acids esterified in membrane phospholipids (Arudini, 1992; Arudini et al., 1995).

Related compounds, such as L-propionyl L-carnitine, have been shown to exhibit antioxidant activity by chelating metals (Reznick et al. 1992) and inhibiting the age-associated increase in lipid peroxidation (Kalaiselvi and Panneerselvam 1998; Rani and Panneerselvam 2002). Experimental data demonstrate an age-associated decrease of tissue levels of L-Carnitine in animals, including humans, and an associated decrease in the integrity of the mitochondrial membrane (Costell & Grisolia, 1989; Liu et al. 2002; Maccari et al. 1990). Since L-Carnitine is essential for translocation of fatty acids into the mitochondrial matrix, it is expected that L-Carnitine deficiency would cause disorders in fatty acid utilization (Iossa et al. 2002). L-Carnitine deficiency is associated with cardiomyopathy (Tripp et al. 1981; Waber et al. 1982), and its administration protects the myocardium against diphtheria toxin, ischemia, myocardial infarction and adriamycin-induced damage (Ferlini et al. 1999).

Data are available concerning brain L-Carnitine status of old rats. One report (Maccari et al. 1990) showed that aged Sprague-Dawley rats (30 months) exhibited a statistically significant decrement of total

* Corresponding author at: Experimental Oncology Department of Oncology, University of Alberta, Cross Cancer Institute, 11560 University Avenue, Edmonton, AB T6G 1Z2, Canada.
E-mail addresses: rahmed@ualberta.ca (R.M. Abou El-Magd), vozza@ualberta.ca (N.F. Vozza), jackt@ualberta.ca (J.A. Tuszynski), dwishart@ualberta.ca (D.S. Wishart).

http://dx.doi.org/10.1016/j.jim.2015.11.006
0022-1759/© 2015 Elsevier B.V. All rights reserved.

L-Carnitine levels in the brain, serum, heart, and tibial muscle, accompanied by a marked increment in the liver. Another report (Hansford and Castro 1982) demonstrated that L-Carnitine levels in muscles of old male Wistar rats were significantly lower than those in young animals.

L-Carnitine was described as an essential nutrient for pre-term babies, certain types (non-ketotic) of hypoglycemics, kidney dialysis patients, cirrhosis, in kwashiorkor, type IV hyperlipidemia, heart muscle disease (cardiomyopathy), and propionic or organic aciduria (acid urine resulting from genetic or other anomalies). In all these conditions and the inborn errors of L-Carnitine metabolism, L-Carnitine is essential to life and L-Carnitine supplements are valuable. L-Carnitine therapy may also be useful in a wide variety of clinical conditions. L-Carnitine supplementation has improved the health status of some patients who presented with angina secondary to coronary artery disease. It may be worth a trial in any form of hyperlipidemia or muscle weakness. L-Carnitine supplements may be useful in many forms of toxic or metabolic liver disease and in cases of heart muscle disease (Scaglia and Longo 1999).

Changes in L-Carnitine metabolism have been demonstrated with alterations in nutritional state and ketosis in animals (Bøhmer 1967; Bøhmer et al. 1966; McGarry and Foster 1977; McGarry et al. 1975; Pearson and Tubbs 1964; Snoswell and Mclntosh 1974) and humans (Frohlich et al. 1978; Genuth and Hoppel 1979, 1981; Maebashi et al. 1977; Maebashi et al. 1978, Maebashi et al. 1976). In diabetic and fasting ketotoic animals, studies have shown alterations in tissue and blood concentrations of free Carnitine and Acylcarnitine (Bøhmer et al. 1966; Brass and Hoppel 1978; Pearson and Tubbs 1967; Snoswell and Mclntosh 1974). Studies in human fasting ketosis and diabetic ketosis have shown a decrease in plasma free L-Carnitine and an increase in the long-chain and short-chain Acylcarnitine (Frohlich et al. 1978; Genuth and Hoppel 1979; Hoppel and Genuth 1980).

L-Carnitine is considered to be a potential biomarker identified and documented in the HMDB database (http://www.hmdb.ca) as lung cancer-related biomarkers (Yang et al. 2010; Wishart et al. 2009).

In addition, a survey of serum L-Carnitine concentrations was made in cancer and non-cancer patients for the purpose of determining the L-Carnitine profile. The acid-soluble Acylcarnitine concentration was significantly lower in cancer patients than in controls (6.7 vs 11.5 nmol/ml). When percentages and ratios were calculated for the relative proportions of Acyl-Carnitines, large variations were found to occur among cancer types. Acyl-Carnitine ratio (the sum of acid-soluble and acid-insoluble acyl-Carnitine divided by non-esterified Carnitine) ranged from 0.17 in leukemia to 0.30 in breast cancer cases. Since Acyl-Carnitine concentration and ratio are reflective of the metabolic state, the depressed Acyl-Carnitine ratio in cancer patients may be due to decreased production, increased utilization, or increased excretion of acid-soluble Acyl-Carnitine. Elevated concentrations of non-esterified L-Carnitine and total L-Carnitine were observed in two patients, and some of the lowest Acyl-Carnitine concentrations and ratios were observed in advanced cancer cases. The therapeutic regimen and/or the neoplastic process itself may be responsible for the observed differences in the serum L-Carnitine profile (Sachan and Dodson 1987).

Phage display has the capacity to rapidly isolate recombinant antibodies against protein targets and other molecules of significant size. The techniques of phage display can be adapted to allow the isolation of antibodies against very small compounds. Antibodies generated in this way have many uses including the detection and quantitative analysis of the target chemical moiety in samples such as foods, water and body fluids (Shaw and Kane 2009).

Single chain Fv (scFv) is defined as a recombinant fusion protein that consists of variable domains from a heavy chain (VH) and a light-chain (VL) connected by an artificial linker (Luo et al. 1997). This small molecule could be used as an in vitro anti-small biomarker antibody for diagnostic purposes. Furthermore, it shows increased accessibility to tumor cells in vivo, and may therefore be a significant method for use in drug, radionuclide and hormone delivery systems (Luo et al. 1996; Wang et al. 2001).

The aim of the present work was to isolate and develop specific (scFv) antibodies for high-value biomarkers of L-Carnitine. The prepared antibody fragment molecules are hoped to primarily provide specific L-Carnitine binding capability to a metabolomic device, whose design and testing are currently underway and which is aimed at diagnostic purposes for multiple diseases using serum and/or urine samples.

2. Materials and methods

The Tomlinson I + J human single fold synthetic naïve phage display single chain antibody fragment libraries (in phagemid/scFv format - fused to the pIII minor coat protein of M13 bacteriophage), helper phage KM13, positive control anti-ubiquitin ScFv in bacterial strain TG1 (labeled TG1-antiubi) *E. coli* strains TG1 and HB2151 for selection of specific antibody clones and for production of soluble single chain scFv, respectively, were obtained from The Medical Research Council (MRC), Cambridge, England (Lee et al. 2007). The size of the library I is 1.47×10^8 phagemid clones in *E. coli* TG1 cells, and has a high proportion of functional antibody fragments with approximately 96% of clones containing inserts. The library J size is 1.37×10^8 with approximately 88% of clones containing inserts. L-Carnitine (HMDB00062), L-Palmitoylcarnitine (HMDB00222) and L-Hexanoylcarnitine (HMDB00756), Trypsin from bovine pancreas and polyethylene Glycol n6.000 were purchased from Sigma (St Louis, MO). Anti-M13 horseradish peroxidase (HRP) conjugate, Protein A horseradish peroxidase (HRP) conjugate, Protein L horseradish peroxidase (HRP) conjugate, ImmunoPureA Purification Kit, were purchased from Pierce, (Rockford, IL, USA). TMB (3.3′,5.5′-tetramethylbenzidine) solution was purchased from Becton Dickinson Biosciences. L-Carnitine is a quaternary ammonium salt, which has a significant ability to adhere to surfaces. For antigen immobilization by absorption on plastic, Nunc MaxiSorp 96-well plates were purchased from VWR Scientific (Nunc cat. no.442404). Isopropyl-beta-D-thiogalactoside (IPTG) was purchased from Gold Biotechnology, USA. For PCR screening process, we used GoTaqG2 Hot Start Colorless Master Mix, which was purchased from Promega, USA.

2.1. Selection of anti L-Carnitine phage clones

The stock libraries I & J and stock helper phage KM13 were first expanded in order to obtain sufficient quantities for future use of several rounds of selections. Each library stock was amplified in 2xTY medium containing 100 μg/ml ampicillin and 4% glucose. Phage particles were rescued by super infection with $2 \times 10^{11\text{-}12}$ phage helper units. Purification of phage repertoire is done using the Poly Ethylene Glycol (PEG) precipitation method (Lee et al. 2007; Marks & Hoogenboom, 1991).

The selection procedures for Libraries I and J were done separately to ensure selecting the most L-Carnitine antigen binding clones. The selection procedure was described previously in detail (Harrison et al. 1996; Chames et al. 2002), with some modifications. In summary, the Maxisorp 96-well plate was coated with 100 μg/ml of L-Carnitine in PBS. The efficiency of coating is highly dependent on the antigen concentration, the buffer and the incubation temperature. Tendency of concentration dependency is highly increased as the absorption capacity of the surface increases upon using higher concentration of L-Carnitine. This greatly enhances the immobilization of the compound towards better efficiency. The quaternary ammonium compounds have been adsorbed on almost any surface resulting in the formation of monolayers. The plate was incubated at 4 °C overnight. 10^{12-13} phage units of the library were mixed together with equal volume of 5% Marvel milk powder (w/v) in PBS final specified concentration, and incubated with end-to-end rotation for 1 h at room temperature, then allowed to stand for a further 1 h. The 96-well plate was washed ten times for the first selection and twenty times for the

following rounds of selections with PBST wash buffers. Finally, bound phages were eluted by adding trypsin (0.1 mg/ml)-PBS and rotating for 10 min (up to 60 min) at room temperature. An aliquot of the eluted phages was used to infect fresh exponentially growing culture of *E. coli* TG1 cells, and incubated at 37 °C in a water bath (without shaking) for 30 min to allow optimal infection. Phage particles for subsequent round of selection were rescued by super infection with helper phage, amplified, and used for further rounds of selection as instructed in the Tomlinson (I + J) protocol. Three rounds of selection experiments were carried out for selection of scFv-phage clones with specific binding to L-Carnitine.

2.2. Polyclonal and monoclonal phage ELISA

Polyclonal phage ELISA was carried out using 50 μl PEG precipitated phage from the end of third round of selection appropriately diluted with 2% MPBS. The diluted phage mixture was incubated in a 96-well plate coated with 0.1 mg/ml of antigen (L-Carnitine) in PBS. The ELISAs were performed essentially as described (Lee et al. 2007). L-Carnitine Bound phage-scFv was detected using 1 in 5000 dilution of HRP-anti-M13 in 2% MPBS (Pierce, (Rockford, IL, USA)) and TMB-ELISA colorimetric assay detection reagents (BD).

In addition, Monoclonal Phage ELISA was carried out by inoculating individual colonies from the titration plates of the third round of selection into 100 μl 2xTY medium containing 100 μg/ml ampicillin and 4% glucose in 96 cell-well plates. The plate was incubated shaking overnight at 37 °C. Then a small inoculum (about 2 μl) from each well was transferred from this plate to a second 96 cell-well plate containing 2xTY with ampicillin and 4% glucose per well. After the optimized time incubation, 10^{10} helper phage was added to each well. After 1 h. incubation, spinning and re-suspending pellet in 2xTY containing 100 μg/ml ampicillin and 50 μg/ml kanamycin, the plate was incubated shaking overnight at 30 °C. 50 μl of the supernatant from each well in the overnight inoculated plate was tested by using phage ELISA.

2.3. Detection of the soluble antibody fragments

Soluble fragments can be produced in *E. coli* HB2151. Glycerol stock of HB2151 bacteria was streaked on M9 minimal medium glucose plate and incubated for 36 h at 37 °C. A single colony was picked and grown overnight in 5 ml 2xTY medium at 37 °C, 250 rpm. This culture was diluted 100-fold into 5 ml of 2xTY medium. Grow at 37 °C, 250 rpm until OD600 = 0.4. 10 μl of eluted phage from the highest titer of the previous experiment with both libraries were inoculated with and infect exponentially growing HB2151 bacteria (OD 600 of 0.4) for 30 min at 37 °C in a water bath. The TYE plates with 100 μg/ml ampicillin and 1% glucose are subcultured 50 μl, 50 μl of $1:10^2$, 50 μl of $1:10^4$, 50 μl of $1:10^6$ dilutions of the infected HB2151 and are incubated overnight at 37 °C. Individual colonies were picked into 100 μl 2xTY 100 μg/ml ampicillin and 1% glucose in 96 cell-well plates and grow shaking (250 rpm) overnight at 37 °C. Small inocula (about 2 μl) were transferred from this plate to a second 96 cell-well plate containing 200 μl 2xTY containing 100 μg/ml ampicillin and 0.1% glucose per well. Grow shaking (250 rpm) at 37 °C until the OD600 is approximately 0.9 (about 3 h). Isopropyl β-D-thiogalactoside; IPTG; induction was done with final concentration 1 mM. 50 μl of the supernatant of the overnight induced plate from each well was transferred to a coated-96 well flexible Maxisorp assay plate with 100 μl-100 μg/ml/well of L-Carnitine for ELISA in 3% BSA-PBS using a 1:5000 dilution of Protein A-HRP or Protein L-HRP to detect binding using TMB reagent as previously mentioned. Flexible Maxisorp format provides flexible use of components with small sample sizes. MaxiSorp plates are hydrophilic and ideal for antibody assays.

2.4. Phagemid DNA sequencing, translation and alignment

Phagemid DNA sequencing of 22 randomly picked clones was performed using the QIA prep Spin M13 Kit (QIAGEN Inc., Valencia, CA). The phagemids were sequenced by the Molecular Biology Services Unit, Centennial Centre for Interdisciplinary Science (CCIS), The University of Alberta. The sequence check was done by using the following primers: pHEN seq [Reverse] (5′ CTA TGCGGC CCC ATT CA 3′), Linker VH Seq. primer [Reverse] (5′ TCC GCC TGA ACC GCC TCC 3′), and RBS-VH Seq. primer: [forward] (5′ AAG GAG ACA GTC ATA ATG 3′). Readily available web based tools were used for translation. The DNAPLOT software was used for alignment of DNA sequences, which was developed by Hans-Helmar Althaus and Werner Müller, and was modified for alignment to V BASE in collaboration with Ian Tomlinson.

2.5. PCR screening for the selected clones

To check individual clones for the presence of full length V inserts. All PCR reactions were carried out using GoTaqG2 Hot Start Colorless Master Mix, LMB3 primer [forward]: (5′ CAG GAA ACA GCT ATG AC 3′), and pHEN seq. primer [Reverse]: (5′ CTA TGC GGC CCC ATT CA 3′). The reaction was done at annealing temperature of 55 °C, 2 min extension for VH and VL together.

2.6. Small-scale expression and purification of soluble Ab fragments

For the production of soluble antibody fragments from selected positive L-Carnitine binding clone, *E.coli* HB2151 non-suppressor strain clones producing the highest amount of soluble Fragments detected in the previous step (D-4-1 clone), were used to inoculate culture flask containing 2xTY/ampicillin/0.1% glucose. The culture was grown with shaking (250 rpm) at 37 °C until the OD600nm was approximately 0.9. IPTG was added to a final concentration of 1 mM. Shaking was continued at 200 rpm, at 30 °C overnight to harvest antibody fragments secreted into culture supernatant and the *E. coli* periplasm by osmotic shock. The induced bacterial culture was centrifuged at 30,000 ×*g* at 4 °C for 30 min and the supernatant containing secreted antibody fragments was collected, and clarified by filtration. The resulting supernatant was concentrated by ammonium sulfate precipitation followed by dialysis [10 kDa cut dialysis system] into binding/loading buffer at 4 °C overnight. The antibody fragments were purified using protein A immobilized on agarose resin (Immunopure kit, according to manufacturer's; Pierce; instructions). To extract the soluble fragments secreted into the *E. coli* cell periplasm, the bacterial pellet from the above centrifugation step was re-suspended in 1/50 the original culture volume of ice-cold 50 mM Tris–HCl, pH 8.0, containing 20% sucrose, 1 mM EDTA (with 1 tablet/50 ml culture of Halt Protease Inhibitor cocktail EDTA-free) The suspension was agitated gently for 30 min in an ice bath or cold room, centrifuged for 30 min at 30,000 ×*g* at 4 °C and the supernatant collected (periplasmic fraction of Ab protein) and maintained at 4 °C. To obtain the osmotic shock fraction, the resulting pellet from the previous periplasm extraction step was again re-suspended in the same volume [1/50] of ice-cold 5 mM MgSO4, 1 mM EDTA, and sufficient volume of Halt Protease Inhibitor. The suspension was agitated gently for 15 min in an ice bath or cold room, centrifuged for 30 min at 30,000 ×*g* at 4 °C, and the supernatant containing the osmotic shock fraction of Ab proteins was collected. Supernatants from both the periplasmic and osmotic shock fractions containing the soluble fraction Ab proteins were collected filtered through disposable 0.45 μm filters and dialyzed (10 kDa cut dialysis system) into binding/loading buffer. Soluble Ab fragments enriched in the periplasmic/osmotic fractions were then purified on ImmunoPure® immobilized protein Acolumns (Pierce). Finally, all eluted protein fractions (supernatant, periplasmic and osmotic shock) were collected and dialyzed to PBS buffer, pH 7.4, and simultaneously concentrated to 1.0–1.5 mL using Amicon 15 ml-30 K to get the effluent then use 10K concentrator

(Pierce). Purity of expressed and extracted soluble Ab fragments was evaluated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The expressed soluble and purified protein from selected clone was then retested using polyclonal ELISA, fluorescence quenching assay as well as Circular Dichroism to determine the binding affinity and ellipticity of the purified soluble fragments.

2.7. Detection of the binding affinity of L-Carnitine soluble antibody fragments using tyrosine fluorescence quenching assay against different antigens

Soluble antibody fragments were mixed with PBS buffer pH 7.4 to reach 1 μM final concentration in a 96-well microplate. L-Carnitine, L-Palmitoylcarnitine, L-Hexanoylcarnitine and Ubiquitine (as a negative control) in PBS were added to the protein samples to obtain final ligand concentrations of 10, 20, 40, 80, 100, 150 and 200 μM. The final volume was 100 μL. A glass bead was inserted into each well, and the microplate was covered with protective film, and incubated for 30 min at 25 °C. After that time, the microplate was transferred to a rotating platform and vigorously rotated for 1 h at room temperature. From each well, 80 μl of samples and control was transferred to a 1-cm fluorescence cell. Fluorescence spectra were collected on a PTI MODEL-MP1 spectrofluorometer using 10 mm path length cell at 280 nm (excitation wavelength), and the scan range was 300–420 nm. Spectral data were collected using fluorescence software, and data analysis was performed using ORIGIN 6.1 software (Origin- Lab., Northampton, MA, USA). Data from the fluorescence experiments were used to determine the apparent binding constant of the selected antigens to the purified antibody fragments using the Stern–Volmer equation (Van de Weert and Stella 2011):

$$\frac{F0-F}{F} = Ka[LA]$$

Where F0 and F are the fluorescence intensities in the absence and in the presence of quencher, Ka is the formation constant of the donor–acceptor (antigen–antibody) complex and [LA] is the concentration of the ligand added. Excitation and emission slits were set at 2 nm. All spectra were collected with samples having final optical densities (1 cm) <0.3 at maximum absorbance of added ligand and were corrected for the inner filter effect. From the slope of the linear plot of ((F0 – F)/F) versus [LA], binding constant values were estimated. The results are expressed as mean values ± SD.

2.8. Circular Dichroism analysis for L-Carnitine soluble antibody fragments

CD spectroscopy was performed on a Jasco J-810 spectropolarimeter. The spectra were recorded using a 1-mm path length cell (equilibrated at room temperature) in a wavelength range from 190 to 260 nm with 1-nm resolution at 20 nm/min scanning speed and 1^{-s} response time. Raw ellipticity data, given in millidegrees (mdeg), was smoothed using the Jasco software, exported, and converted to molar ellipticity, [θ]. To convert from mdeg to molar ellipticity [θ] in deg. cm2/dmol, Equation 1 (Greenfield 2007) was used,

$$[\theta] = \frac{(Mdeg\ x\ MRW)}{Pathlength\ x\ [Carnitine\ sdAb]}$$

Where the mean residue weight, *MRW* = (molecular weight of the antibody in Da/number of backbone amino acids), *Pathlength* = cell path length in mm, and [*Carnitine sdAb*] = concentration of antibody in mg/ml.

3. Results

3.1. Polycolonal and monoclonal phage ELISA

In these experiments, phage was quantified by tittering after each round of the three selection experiments. The concentration of phage used for each round of selection was constant at $2 \times 10^{11-12}$ particles/ml. The phage count titration is measured according to the empirical equation = titer phage/ml × OD260 nm × 100 × 22.14 × 10^{10}.

The results showed that the relative eluate yield of binding phage particles was increased significantly from the first to the third round of affinity selection for libraries I (1.9×10^{12} to 3.18×10^{13}) and J (1.3×10^{12} to 3.5×10^{13}), respectively. These results clearly indicate that isolation and enrichment of L-Carnitine binding phages was accomplished.

Polyclonal phage ELISA was carried out using 50 μl PEG precipitated phage from the end of third round of selection. The results showed that the selectivity for the phages separated from both library I and J is significantly high (Fig. 1-A) using 100 μg/ml of L-Carnitine antigen and Ubiquitine as an antigenic negative control. The absorbance difference for the library I-selected phage for L-Carnitine was 0.06–0.07 and for Library J-selected phage was 0.07–0.08 comparing to Ubiquitine absorbance which was 0.003 for both library I and J, respectively.

Monoclonal Phage ELISA was carried out by inoculating individual colonies from the titration in 96-well plates of the third round of selection. The results of the monoclonal phage ELISA show that positive clones, shown in Fig. 1-B, with specific binding to adsorbed L-Carnitine as an antigen on the surfaces of the wells have been selected from both Tomlinson I + J libraries. As illustrated in Fig. 1-B-1. The absorbance difference for the library I-selected phage clone D-4 for L-Carnitine was 0.06–0.07. In Fig. 1-B-2, and for Library J-selected phage H(8)-1 was 0.14–0.15 Ubiquitine absorbance which was 0.005–0.006 for both library I and J respectively.

3.2. Detection of the soluble antibody fragments

In the monoclonal Phage ELISA experiment, it has been illustrated that the clones D-4 from Library I and Clone H-8(1) from Library J have a significant selectivity for L-Carnitine phage/scFv fragment selectivity. Soluble fragments were produced by transforming the selected clones to *E. coli* HB2151. IPTG induction was done with final concentration 1 mM. 50 μl of the supernatant of the overnight induced plate from each well was transferred to a coated-96 well flexible Maxisorp assay plate with 100 μl-100 μg/ml per well of L-Carnitine for ELISA in 3% BSA-PBS using a 1:5000 dilution of Protein A-horse radish peroxidase; (HRP) or Protein L-HRP to detect binding using TMB reagent. Our results revealed that the soluble antibody fragments which are produced from Library I are much more selective and better detected by protein A than the antibody fractions which were produced by library J. As illustrated in Fig. 2, a significant increase in the mean absorbance difference for the library I soluble fragments upon using protein A as a detector for the soluble antibody fragments (0.34) than using protein L (0.1), respectively. On the other hand, there is a significant increase in the mean absorbance difference for the the library J soluble fragments upon using protein A (0.25) than when using protein L (0.05), respectively.

3.3. Phagemid DNA sequencing, translation and alignment

The phagemid DNA sequences were examined on the selected L-Carnitine-binding scFv clones to check the structure if they have full length VH and VL inserts or not. Of the 22 phage clones randomly picked for sequencing, all clones from Tomlinson I libraries had distinct amino acid sequences (Fig. 3-A; B). The translation of the DNA sequences into amino acid sequences and subsequent protein

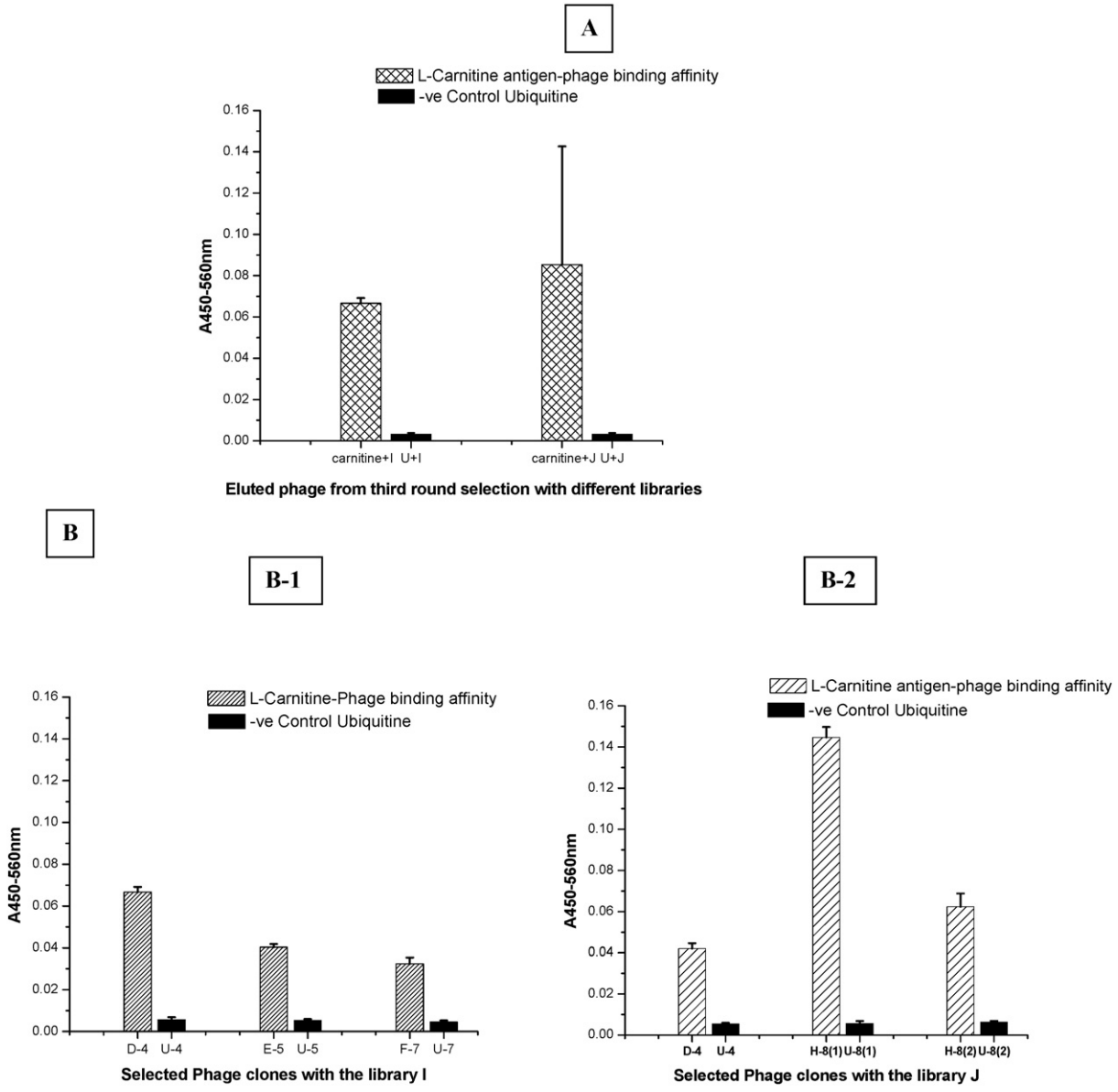

Fig. 1. A: polyclonal phage ELISA determined binding properties of L-Carnitine selected phage from Tomlinson I & J libraries. L-Carnitine Antigen concentration used in selection was 100 μg/ml. Data represent mean ± standard deviation from triplicate measurements using Ubiquitine as –ve control. B: Monoclonal phage ELISA: B-1: Monoclonal phage ELISA determined binding properties of L-Carnitine selected phage best selective clones D-4, E-5, F-7 from Tomlinson I library. L-Carnitine Antigen concentration used in selection was 100 μg/ml. Data represent mean ± standard deviation from triplicate measurements using Ubiquitine as –ve control. B-2: Monoclonal phage ELISA determined binding properties of Carnitine selected phage best selective clones D-4, H-8(1), H-8(2) from Tomlinson J library. L-Carnitine Antigen concentration used in selection was 100 μg/ml. Data represent mean ± standard deviation from triplicate measurements using Ubiquitine as –ve control.

alignment were accomplished using readily available free web-based tools: (http://web.expasy.org/translate/;http://www.vbase2.org/vbase2_search.php).

The results show that the clones have a complete VL sequence attached to the designed linker but missing a new insert for VH sequence other than the short one for the original library design. Nucleotide sequences or amino acid inserts were used for further studies. VL sequence contains 101 amino acids with 93.06% homology among the whole VL sequences for C-4-1, 2, 3, 4 and 6 clones. However, D-4-5 has 92.07% homology, as illustrated in (Fig. 3-B). The CDR regions are detected and the length of CDR1, CDR2 and CDR3 for the clones is the same. D-4-1 clone is expressed in soluble form for Small-scale expression and purification of soluble Ab fragments.

Deduced amino acid sequences (including depiction of CDR) of soluble fragments with binding specificity to L-Carnitine are isolated from Tomlinson I Library. This alignment was done by using the DNAPLOT software to align the nucleotide sequence of the rearranged V gene to its closest germline segment counterparts.

3.4. PCR screening for the selected clones

Another way to investigate the individual clones for the presence of full length V inserts is to carry out PCR reaction screening. These reactions were carried out using LMB3 primer [forward] and pHEN seq. primer [Reverse], in order to check the presence and/or absence of both VH and VL. Our data revealed that the six clones give PCR products around 650–700 bp, which correspond to one V insert in addition to the linker as illustrated in (Fig. 4). According to the Tomlinson protocol, PCR product size = 935 pb corresponds to the presence of

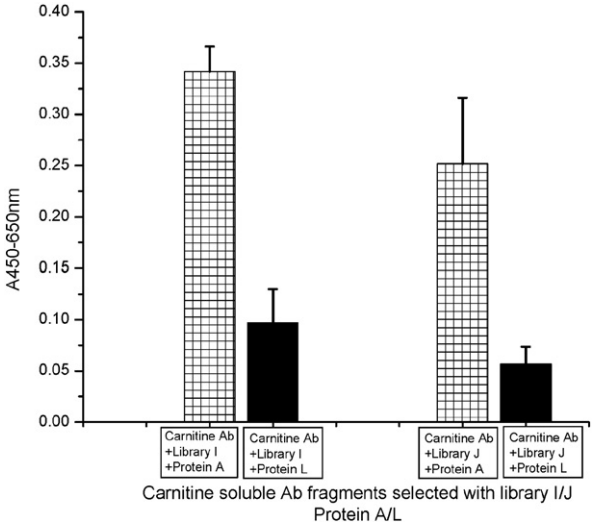

Fig. 2. Monoclonal ELISA determined binding properties of Carnitine selected soluble fragments produced from D-4 clone from Tomlinson I & H-8(1) clone from J libraries using Horse radish peroxidase conjugated protein A and L. L-Carnitine Antigen concentration used in selection was 100 μg/ml. Data represent mean ± standard deviation(n = 4).

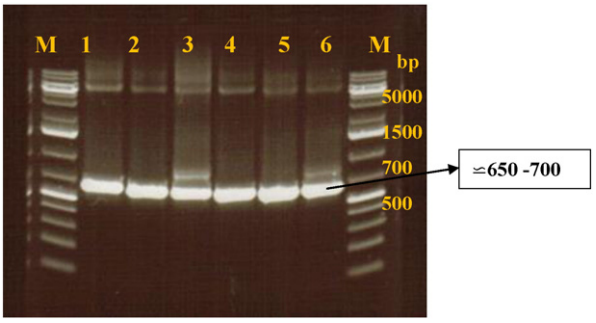

Fig. 4. Agarose gel electrophoresis analysis of VL and VH assembly. Lane M, 1Kb plus DNA ladder marker; lane 1–6, reaction products from the PCR using the purified D4-1-6 phagemide.LMB3 primer (forward): CAG GAA ACA GCT ATG AC & pHEN seq primer (reverse): CTA TGC GGC CCC ATT CA; annealing temperature = 55 °C for 2 min extension for VH and VL together. For VH and VL together, the insert should 935 bp In our PCR product with insert = 650–700 bp, without insert = 329 bp.

both VH and VL insert, while 329 pb corresponds to the absence of both of the V inserts.

3.5. Small-scale expression and purification of soluble Ab fragments

Highly purified 1.3 mg/L of antibody soluble portions were obtained in the form of the 98% purified soluble ScFv fragments (SDS-PAGE 12%), through small scale expression and multi-step purification of the eluted protein fractions (supernatant, periplasmic and osmotic shock). As illustrated in Fig. 5-A, a very clear band of around 16 kD of the purified antibody fragment was detected as a VL-linker soluble fraction. Molecular weights can be affected by complementarily determining region (CDR) length. Dimerization may occur forming higher molecular weight detected bands as seen around 32 kD.

In addition, to investigate the selectivity of the purified Ab fraction to L-carnitine antigen, polyclonal ELISA determined binding properties of L-Carnitine specific soluble fragments (secreted, periplasmic and osmotic shock portions). As illustrated in Fig. 5-B, a significant increase in the absorbance difference (the indicator for the efficiency of the selectivity of the Ab fragments) to reach 0.86 for the secreted soluble Ab fragments and 0.72 for the periplasmic and osmotic shock eluted soluble Ab fragments against L-Carnitine as the selected antigen. Very

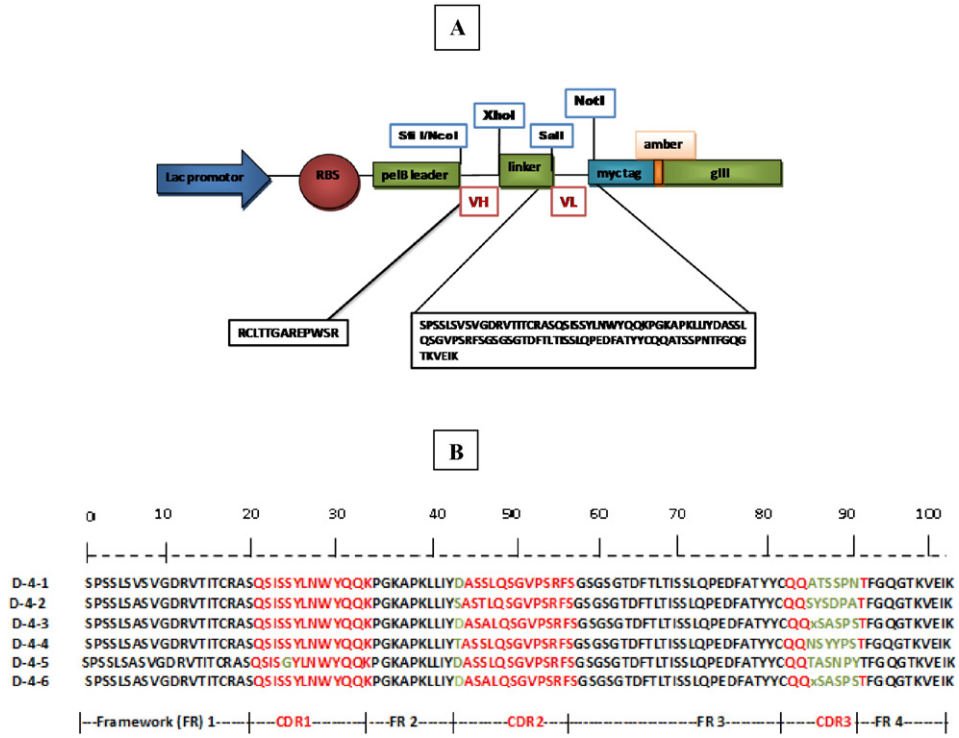

Fig. 3. A: Illustrative DNA sequences of the L-Carnitine selective U-4-1 clone with the sequence of inserted V_L and absence of any new insertion for V_H fragment other than the short insertion in the provided Tomlinson Library with pIT2 vector. B: Sequence analysis for VL fragment of anti-L-Carnitine soluble scFv. Six sequences were selected among the 40 anti-L-Carnitine binders based on the Vbase2 software. D-4-1, 2, 3, 4 and 6 have 93.06% homology in the overall sequence. D-4-5VL clone exhibits approximately 92.07% homology in sequence with the previous clones. The length of CDR1, CDR2and CDR3 for the clones is the same. Black sequence has 100% homology. Red sequence corresponds to the CDR regions and green sequence corresponds to sequence differences.

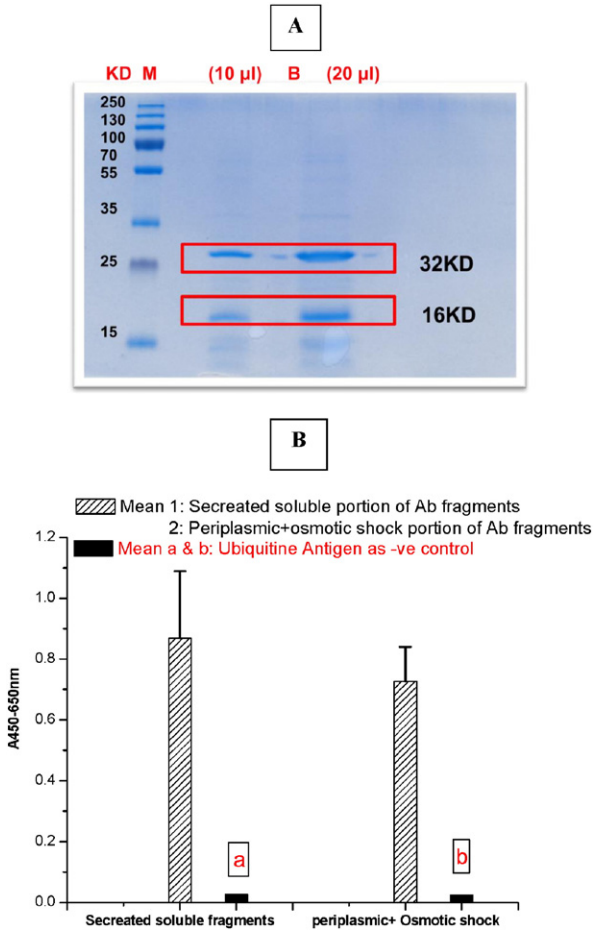

Fig. 5. A: Analysis of sample purity by SDS-PAGE: One microgram of soluble D-4 clone expressed Ab fragments (10 & 20 µl) were loaded in each well of a 12% SDS-PAGE. Expected molecular weights: VL, 16 k Da. Molecular weights can be affected by complementarity determining region (CDR) length. Dimerization may occur forming higher molecular weight detected bands. B: polyclonal ELISA determined binding properties of L-Carnitine specific soluble fragments (secreated, periplasmic and osmotic shock portions) during small-scale production of the Ab fragments. L-Carnitine Antigen concentration used in selection was 100 µg/ml. Ubiquitine antigen was used as negative control for binding efficiency. Data represent mean ± standard deviation from triplicate measurements.

low, almost no selectivity has been detected for the same ELISA test using Ubiquitine as negative control for the selective antigen.

3.6. Detection of the binding affinity of L-Carnitine soluble antibody fragments using tyrosine fluorescence quenching assay against different antigens

Results of the monoclonal phage/scFv ELISA and polyclonal Phage/ScFv clearly indicate that there is significant affinity of the antibody fragments to L-Carnitine antigen.

To confirm the efficiency and selectivity of the purified soluble L-Carnitine Ab fraction, we examined the binding affinity of the Ab fragments using tyrosine fluorescence quenching biophysical assay. The presence of five tyrosine residues in the VL fragment was confirmed by the sequence check. The binding affinity to L-Carnitine and L-Hexanoylecarnitine as positive, strong chemically related compounds was confirmed. In addition, other antigens were tested to check the selectivity of the Ab, those antigens are:L-Palmitoylcarnitine (Fig. 6-C) whose structure is chemically related to the main L-Carnitine molecule except for the presence of a very long aliphatic side chain, and Ubiquitine (as a negative control; see Fig. 6-D). L-Carnitine showed a strong binding affinity while L-Hexanoylecarnitine showed a lower affinity compared with L-Carnitine when tested with a tyrosine fluorescence quenching assay towards the purified soluble fractions of L-Carnitine antibody. The binding induces a conformational change in the antibody fragments as illustrated in (Fig. 6; A-B). To specify the binding affinity to each of the tested antigens, our data revealed that the Kd values, which correspond to the dissociation constant and are used to identify the binding affinity are 0.8 µM and 1 µM for L-Carnitine and L-Hexanoylecarnitine, respectively. The characteristic tyrosine fluorescence emission spectrum, which presents in the VL of the antibody, was significantly quenched by L-Carnitine. No binding has been detected when L-Palmitoylcarnitine or Ubiquitine were used as antigens indicating there is neither affinity nor selectivity for both of the antigens, respectively. These fluorescence quenching studies indicated that the purified antibody fragments work at a very high selectivity and affinity towards the desired antigen. Analysis of the secondary structure of the purified L-Carnitine soluble Ab by Circular Dichroism spectroscopy (CD) (Fig. 7) showed that the Ab fragment represents a folded protein that mainly consists of β-sheets. The obtained pattern corresponds to the typical secondary structure of functional ScFvs (Umetsu et al. 2003; Correcirc and Ramos 2009; Lupinek et al. 2009).

4. Discussion

The main purpose for using Tomlinson A and J libraries was to select the phage which produces an scFv fragment that specifically recognizes and attaches to L-Carnitine. Surprisingly we obtained soluble antibody fragments, which consist of a VL insert, linker but without a VH insert. Through the screening process for the selected clones, the data confirmed that the structure of the scFv for L-Carnitine contains only a VL fragment attached to the linker.

Having consulted the providing company (Life Sciences on behalf of the MRC Laboratory of Molecular Biology and the MRC Centre for Protein Engineering, Cambridge, UK), it was confirmed that the effect of getting these soluble fragments is other than getting scFv fragments. It is not uncommon that the scFv constructs are missing either a VH or a VL domain. This would have occurred during the construction of the library, and the library originators found this phenomenon, too. This does not prevent antigen binding, and specific binders of just VH or VL domains were seen by the originators.

L-Carnitine is a Cationic quaternary amine that occurs naturally in most mammalian tissue. In cationic surfactants, a cation is the surface-active species. The majority of cationic surfactants are based on the nitrogen atom carrying the cationic charge. Both amine and quaternary ammonium-based products are common. The amines only function as a surfactant in the protonated state; therefore, they cannot be used at high pH values. Quaternary ammonium compounds, 'quats' on the other hand, are not pH sensitive. The majority of the applications for quaternary ammonium cpds are related to their very strong affinity for surfaces, which makes them powerful surfactants (Malik et al. 2011). The quaternary ammonium compounds have been adsorbed on almost any surface resulting in the formation of monolayers. Depending on the unique ability for L-Carnitine to adhere to surfaces, MaxiSorp plates were used in the selection steps in addition to ELISA tests in order to immobilize the antigen without using any other chemically modified reaction such as Biotinylation.

Binding efficiency is measured as the fraction of input phage that binds the target molecule. The results obtained showed that the relative eluate yield of binding phage particles was increased from the first to the third round of selection through successful selection experiments. The results clearly indicate that isolation and enrichment of L-Carnitine binding phages was accomplished.

Phage antibody selections involve the sequential enrichment of a specific binding phage from a large excess of non-binding clones. This is achieved by multiple rounds of phage binding to the target antigen, washing to remove non-specific phage and elution to retrieve specific

binding. Any method that separates clones that bind from those that do not can be used as a selection method. Many different selection methods have been used. The most popular procedure, which has been proved to be highly sensitive and efficient, is the one we used in our method, selection on immobilized antigen coated onto solid support. Other selection methods are used in other studies including columns or BIAcore sensor chips (Clackson et al. 1991; Marks et al. 1991; Griffiths et al. 1994; Malmborg et al. 1996), selection using

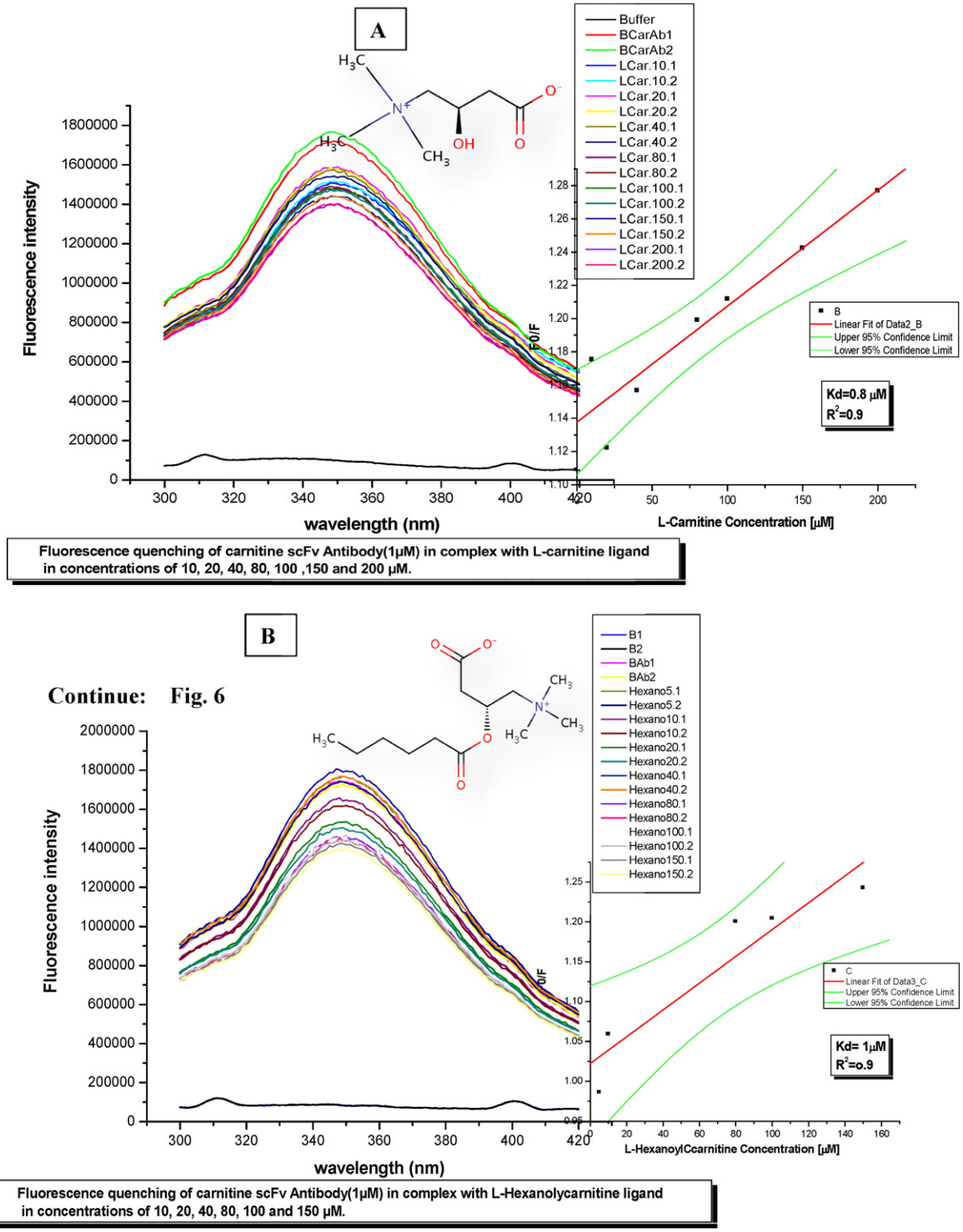

Fig. 6. Soluble antibody fragments were mixed with PBS buffer pH 7.4 to reach 1 µM final concentration in a 96-well microplate. The calculated amounts of stock solution of the compounds which were used as antigens, including L-Carnitine (Fig. 6-A), L-Hexanoylcarnitine (Fig. 6-B), L-Palmitoylcarnitine (Fig. 6-C), and Ubiquitine (as a negative control; (Fig. 6-D) in PBS were added to the protein samples to obtain final ligand concentrations of 10, 20, 40, 80, 100, 150 and 200 µM and the final volume was 100 µl. Fluorescence spectra were collected on a PTI MODEL-MP1 spectrofluorometer using 10 mm path length cell at 280 nm (excitation wavelength), and the scan range was 300–420 nm. Spectral data were collected using fluorescence software, and data analysis was performed using ORIGIN 6.1 software.

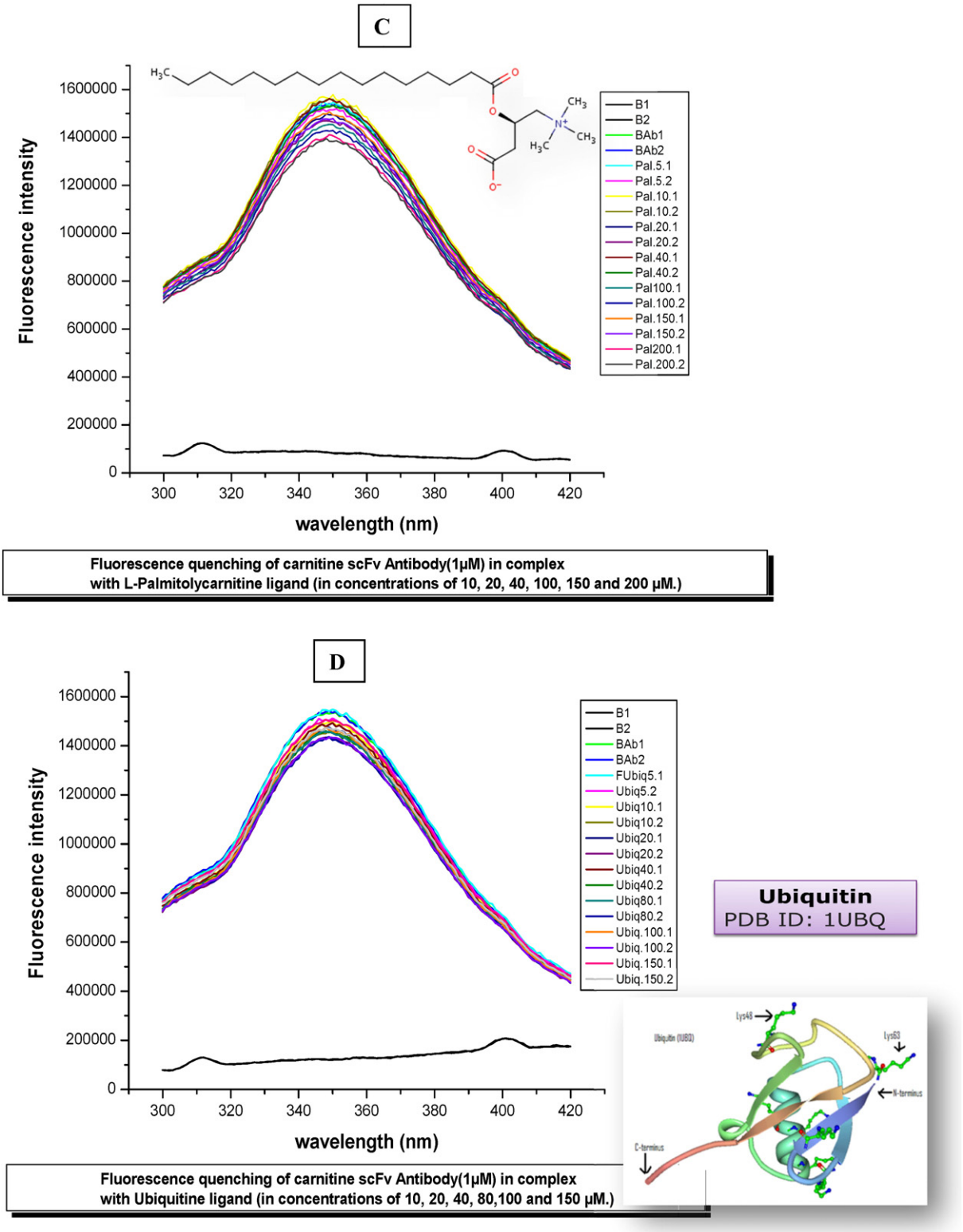

Fig. 6 (*continued*).

biotinylated antigen (Hawkins et al. 1992), panning on fixed prokayotic cells (Hanes and Plückthun 1997) and on mammalian cells (Cai and Garen 1995).

The phagemid DNA sequences were examined to investigate if the selected L-Carnitine binding scFv clones have full length VH and VL or not. The results illustrated using the phagemid DNA sequence check; PCR product screening and the size of the purified soluble Ab fragment separated on SDS-PAGE confirmed that all the selected clones incorporating one V segment, which is VL insert (Fig. 3).

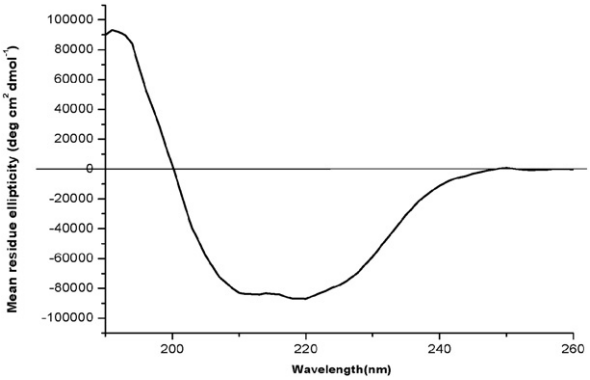

Fig. 7. The Circular Dichroism spectrum of L-Carnitine ScFv was recorded in a wavelength range from 190 to 260 nm (x-axis) and is expressed as mean residue ellipticity (y-axis).

Based on the above results, we selected some of the positive L-Carnitine binding clones for small scale production of soluble scFv antibody fragments in the non-suppressor HB2151 *E. coli* strain according to the Tomlinson library protocol. Soluble scFv fragments protein expression levels for these clones were in the range 1.1 to 1.3 mg/l of culture. These yields are in general agreement with published yields in the range of 0.1 to 5.0 mg per liter of *E. coli* culture for clones isolated from the Tomlinson libraries (Kennel et al. 2004; Wu et al. 2007; Lobova et al. 2008).

The outcome of any selection procedure is a mixture of binding ligands with differing properties. It may be necessary to screen the purified antibodies to identify those variants with the most optimal characteristics. The best screening assay that could be used in this condition is, ELISA; as it is fast, robust, sensitive and amenable to automation. The purpose of these ELISA experiments was to determine whether the soluble scFv proteins would still recognize and bind to L-Carnitine epitopes after expression and purification (that is, in the absence of the phage particles to which they were fused during initial screening and monoclonal fusion phage/scFv ELISA experiments). The results of the polyclonal phage and followed by monoclonal phage/scFv ELISA revealed that positive clones with highly specific binding to immobilized L-Carnitine antigen have been selected from both Tomlinson I + J libraries. ELISA has become extraordinarily useful because it allows rapid screening and quantitation of a large number of samples for the presence of the desired antigen or the antibody recognizing it. ELISA, however, remains popular because of its ease of performance and automation, accuracy, and the ready availability of inexpensive reagents. An alternative approach with ease-of-use and suitable for screens with multiple conditions or clones screened simultaneously is the Thermal Shift Assay (TSA) (Niesen et al. 2007). For the clones derived from D-4 in this study, TSA assays were done to determine if all clones would have similar biding capacities. Although clones from D-4 (1 to 6) showed differential shifts in response to Carnitine (not shown), quantitative results are not readily available and Clone D-4-1 was chosen for further testing due to higher expression levels and solubility.

Five tyrosine amino acid molecules have been detected in the VL segment of all the purified and sequenced checked phagemides. Tyrosine is often regarded as a rather simple fluorophore. However, under some circumstances tyrosine can also display complex spectral properties. Tyrosine can undergo excited-state ionization, resulting in the loss of the proton on the aromatic hydroxyl group. This component is shown in the difference emission spectrum that displays a maximum near 345 nm. The important point is that the phenolic group of tyrosine can ionize even at neutral pH, and the extent to which this occurs depends on the base concentration and exposure of tyrosine to the aqueous phase. Quenching of tyrosine by phosphate and other bases can thus proceed by both static complex formation and by a collisional Stern-Volmer process (Alev-Behmoaras et al. 1979; Schnarr and Helene 1982).

In addition to tyrosine quenching technique used for screening, Circular Dichroism (CD) was used to determine the extent of purity and structure of the purified Ab fragments. One of the main applications of CD for the study of proteins is the estimation of secondary structure of them. Our highly purified soluble antibody fragment was well-behaved when conventional CD analysis was used on far-UV spectrum. The spectra were collected from 190 to 250 nm.The spectrum for L-Carnitine Ab translated as β sheet protein, which has a negative band between 210 and 220 nm and a positive band between 195 and 200 nm. (Correcirc and Ramos 2009).

5. Conclusion

We have developed a soluble antibody fragment that, due to its high affinity and specificity to small molecule L-Carnitine, could be used as a tool to elucidate the role of L-Carnitine in health and disease and to develop new therapeutic agents and/or diagnostic tools for L-Carnitine-mediated diseases.

Phage display is a well-established approach for the identification of bioactive peptides and antibody fragments from large, highly diverse libraries. The specificity, selectivity, and potency of peptides and antibodies discovered in this way may have clinical advantages over traditional small molecule chemotherapeutics, and "targeted therapeutics" is a growing area (Kupsch et al. 1999; Brissette et al. 2006; Almagro and Fransson 2008; Lonberg 2008).

Naïve libraries have been used for isolation of anti-antigen antibodies, but they generally yield lower affinity antibodies than needed for sensitive assay development. We managed to purify high-affinity soluble antibody fragments from naïve libraries. An in-vitro affinity maturation step may be required to increase the affinity of the purified Ab (Shaw and Kane 2009).

Acknowledgments

This work was partly funded by Alberta Innovates – Health Solutions within the framework of the CRIO Team project "Metabolomic Devices for Companion Diagnostics & Personalized Medicine" awarded to DSW.

References

Alev-Behmoaras, T., Toulme, J.J., Hélène, C., 1979. Quenching of tyrosine fluorescence by phosphate ions: a model study for protein-nucleic acid complexes. Photochem. Photobiol. 30 (5), 533–539.
Almagro, J.C., Fransson, J., 2008. Humanization of antibodies. Front. Biosci. 13, 1619–1633.
Arduini, A., 1992. Carnitine and its acyl esters as secondary antioxidants? Am. Heart J. 123 (6), 1726–1727.
Arduini, A., Dottori, S., Molajoni, F., Kirk, R., Arrigoni-Martelli, E., 1995. The Carnitine System. Springer, Netherlands, pp. 169–181.
Bøhmer, T., 1967. Tissue levels of activated fatty acids (acylcarnitines) and the regulation of fatty acid metabolism. Biochim. Biophys. Acta, Lipids Lipid Metab. 144 (2), 259–270.
Bøhmer, T., Norum, K.R., Bremer, J., 1966. The relative amounts of long-chain acylcarnitine, acetylcarnitine, and free carnitine in organs of rats in different nutritional states and with alloxan diabetes. Biochim. Biophys. Acta, Lipids Lipid Metab. 125 (2), 244–251.
Brass, E.P., Hoppel, C., 1978. Carnitine metabolism in the fasting rat. J. Biol. Chem. 253 (8), 2688–2693.
Brissette, R., Prendergast, J.K., Goldstein, N.I., 2006. Identification of cancer targets and therapeutics using phage display. Curr. Opin. Drug Discovery Dev. 9 (3), 363.
Cai, X., Garen, A., 1995. Anti-melanoma antibodies from melanoma patients immunized with genetically modified autologous tumor cells: selection of specific antibodies from single-chain Fv fusion phage libraries. Proc. Natl. Acad. Sci. 92 (14), 6537–6541.
Chames, P., Hoogenboom, H.R., Henderikx, P., 2002. Selection of antibodies against biotinylated antigens. Antibody Phage Display. Humana Press, pp. 147–157.
Clackson, T., Hoogenboom, H.R., Griffiths, A.D., Winter, G., 1991. Making antibody fragments using phage display libraries. Nature 352 (6336), 624–628.
Correcirc, D.H., Ramos, C.H., 2009. The use of circular dichroism spectroscopy to study protein folding, form and function. Afr. J. Biochem. Res. 3 (5), 164–173.
Costell, M., O'Connor, J.E., Grisolia, S., 1989. Age-dependent decrease of carnitine content in muscle of mice and humans. Biochem. Biophys. Res. Commun. 161 (3), 1135–1143.

Ferlini, C., De Angelis, C., Biselli, R., Distefano, M., Scambia, G., Fattorossi, A., 1999. Sequence of metabolic changes during X-ray-induced apoptosis. Exp. Cell Res. 247 (1), 160–167.
Frohlich, J., Seccombe, D.W., Hahn, P., Dodek, P., Hynie, I., 1978. Effect of fasting on free and esterified carnitine levels in human serum and urine: correlation with serum levels of free fatty acids and β-hydroxybutyrate. Metabolism 27 (5), 555–561.
Genuth, S.M., Hoppel, C.L., 1979. Plasma and urine carnitine in diabetic ketosis. Diabetes 28 (12), 1083–1087.
Genuth, S.M., Hoppel, C.L., 1981. Acute hormonal effects on carnitine metabolism in thin and obese subjects: responses to somatostatin, glucagon, and insulin. Metabolism 30 (4), 393–401.
Greenfield, N.J., 2007. Using circular dichroism spectra to estimate protein secondary structure. Nat. Protoc. 1 (6), 2876–2890.
Griffiths, A.D., Williams, S.C., Hartley, O., Tomlinson, I.M., Waterhouse, P., Crosby, W.L., Kontermann, E., Jones, P.T., Low, N.M., Allison, T.J., Prospero, T.D., Hoogenboom, H.R., Nissim, A., Cox, J.P.L., Harrison, J.L., Zaccolo, M., Gherardi, E., Winter, G., 1994. Isolation of high affinity human antibodies directly from large synthetic repertoires. EMBO J. 13, 3245–3260.
Hanes, J., Plückthun, A., 1997. In vitro selection and evolution of functional proteins by using ribosome display. Proc. Natl. Acad. Sci. 94 (10), 4937–4942.
Hansford, R.G., Castro, F., 1982. Age-linked changes in the activity of enzymes of the tricarboxylate cycle and lipid oxidation, and of carnitine content, in muscles of the rat. Mech. Ageing Dev. 19 (2), 191–200.
Harrison, J.L., Williams, S.C., Winter, G., Nissim, A., 1996. [5] Screening of phage antibody libraries. Methods Enzymol. 267, 83–109.
Hawkins, R.E., Russell, S.J., Winter, G., 1992. Selection of phage antibodies by binding affinity: mimicking affinity maturation. J. Mol. Biol. 226 (3), 889–896.
Hoppel, C.L., Genuth, S.M., 1980. Carnitine metabolism in normal-weight and obese human subjects during fasting. Insulin 20, 10.
Iossa, S., Mollica, M.P., Lionetti, L., Crescenzo, R., Botta, M., Barletta, A., Liverini, G., 2002. Acetyl-L-carnitine supplementation differently influences nutrient partitioning, serum leptin concentration and skeletal muscle mitochondrial respiration in young and old rats. J. Nutr. 132 (4), 636–642.
Kalaiselvi, T., Panneerselvam, C., 1998. Effect of L-carnitine on the status of lipid peroxidation and antioxidants in aging rats. J. Nutr. Biochem. 9 (10), 575–581.
Kennel, S.J., Lankford, T., Foote, L., Wall, M., Davern, S., 2004. Phage display selection of scFv to murine endothelial cell membranes. Hybrid. Hybridomics 23 (4), 205–211.
Kupsch, J.M., Tidman, N.H., Kang, N.V., Truman, H., Hamilton, S., Patel, N., ... Crowe, J.S., 1999. Isolation of human tumor-specific antibodies by selection of an antibody phage library on melanoma cells. Clin. Cancer Res. 5 (4), 925–931.
Lee, C.M., Iorno, N., Sierro, F., Christ, D., 2007. Selection of human antibody fragments by phage display. Nat. Protoc. 2 (11), 3001–3008.
Liu, J., Atamna, H., Kuratsune, H., Ames, B.N., 2002. Delaying brain mitochondrial decay and aging with mitochondrial antioxidants and metabolites. Ann. N. Y. Acad. Sci. 959 (1), 133–166.
Liu, J., Head, E., Kuratsune, H., Cotman, C.W., Ames, B.N., 2004. Comparison of the effects of L-Carnitine and Acetyl-L-Carnitine on carnitine levels, ambulatory activity, and oxidative stress biomarkers in the brain of old rats. Ann. N. Y. Acad. Sci. 1033 (1), 117–131.
Lobova, D., ČíŽek, A., Celer, V., 2008. The selection of single-chain Fv antibody fragments specific to Bhlp 29.7 protein of *Brachyspira hyodysenteriae*. Folia Microbiol. 53 (6), 517–520.
Lonberg, N., 2008. Human monoclonal antibodies from transgenic mice. Therapeutic Antibodies. Springer, Berlin Heidelberg, pp. 69–97.
Luo, D., Mah, N., Krantz, M., Wishart, D., Jacobs, F., Martin, L., 1997. High level secretion of single-chain antibody in Pichia expression system. Biotechnol. Tech. 11 (10), 759–761.
Luo, D., Mah, N., Wishart, D., Zhang, Y., Jacobs, F., Martin, L., 1996. Construction and expression of bi-functional proteins of single-chain Fv with effector domains. J. Biochem. 120 (2), 229–232.
Lupinek, C., Roux, K.H., Laffer, S., Rauter, I., Reginald, K., Kneidinger, M., ... Valenta, R., 2009. Trimolecular complex formation of IgE, FcεRI, and a recombinant nonanaphylactic single-chain antibody fragment with high affinity for IgE. J. Immunol. 182 (8), 4817–4829.
Maccari, F., Arseni, A., Chiodi, P., Ramacci, M.T., Angelucci, L., 1990. Levels of carnitines in brain and other tissues of rats of different ages: effect of acetyl-L-carnitine administration. Exp. Gerontol. 25 (2), 127–134.
Maebashi, M., Kawamura, N., Sato, M., Imamura, A., Yoshinaga, K., Suzuki, M., 1977. Urinary excretion of carnitine in patients with hyperthyroidism and hypothyroidism: augmentation by thyroid hormone. Metabolism 26 (4), 351–356.
Maebashi, M., Kawamura, N., Sato, M., Yoshinaga, K., Suzuki, M., 1976. Urinary excretion of carnitine in man. J. Lab. Clin. Med. 87 (5), 260–266.
Maebashi, M., Sato, M., Kawamura, N., Imamura, A., Yoshinaga, K., 1978. Lipid-lowering effect of carnitine in patients with type-IV hyperlipoproteinaemia. Lancet 312 (8094), 805–807.
Malik, M.A., Hashim, M.A., Nabi, F., AL-Thabaiti, S.A., Khan, Z., 2011. Anti-corrosion ability of surfactants: a review. Int. J. Electrochem. Sci. 6 (6), 1927–1948.
Malmborg, A.C., Dueñas, M., Ohlin, M., Söderlind, E., Borrebaeck, C.A., 1996. Selection of binders from phage displayed antibody libraries using the BIAcore™ biosensor. J. Immunol. Methods 198 (1), 51–57.
Marks, J., Hoogenboom, H., Bonnert, T., McCafferty, J., Griffiths, A., Winter, G., 1991. Human antibodies from phage display libraries. J. Mol. Biol. 222, 581–597.
McGarry, J.D., Foster, D.W., 1977. Hormonal control of ketogenesis: biochemical considerations. Arch. Intern. Med. 137 (4), 495–501.
McGarry, J.D., Robles-Valdes, C., Foster, D.W., 1975. Role of carnitine in hepatic ketogenesis. Proc. Natl. Acad. Sci. 72 (11), 4385–4388.
Niesen, F.H., Berglund, H., Vedadi, M., 2007. The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability. Nat. Protoc. 2 (9), 2212–2221.
Pearson, D.J., Tubbs, P.K., 1964. Tissue levels of acid-insoluble carnitine in rat heart. Biochim. Biophys. Acta 84 (6), 772–773.
Pearson, D.J., Tubbs, P.K., 1967. Carnitine and derivatives in rat tissues. Biochem. J. 105, 953–963.
Rani, P.J.A., Panneerselvam, C., 2002. Effect of L-carnitine on brain lipid peroxidation and antioxidant enzymes in old rats. J. Gerontol. Ser. A Biol. Med. Sci. 57 (4), B134–B137.
Reznick, A.Z., Kagan, V.E., Ramsey, R., Tsuchiya, M., Khwaja, S., Serbinova, E.A., Packer, L., 1992. Antiradical effects in L-propionyl carnitine protection of the heart against ischemia–reperfusion injury: the possible role of iron chelation. Arch. Biochem. Biophys. 296 (2), 394–401.
Sachan, D.S., Dodson, W.L., 1987. The serum carnitine status of cancer patients. J. Am. Coll. Nutr. 6 (2), 145–150.
Scaglia, F., Longo, N., 1999, April. Primary and secondary alterations of neonatal carnitine metabolism. Seminars in Perinatology Vol. 23. WB Saunders, pp. 152–161.
Schnarr, M., Helene, C., 1982. Effects of excited-state proton transfer on the phosphorescence of tyrosine–phosphate complexes. Photochem. Photobiol. 36 (1), 91–93.
Shaw, I., Kane, M., 2009. The isolation of scFvs against small target molecules. Antibody Phage Display. Humana Press, pp. 83–97.
Snoswell, A.M., McIntosh, G.H., 1974. The liver as the site of carnitine biosynthesis in sheep with alloxan-induced diabetes. Aust. J. Biol. Sci. 27 (6), 645–650.
Tripp, M.E., Katcher, M.L., Peters, H.A., Gilbert, E.F., Arya, S., Hodach, R.J., Shug, A.L., 1981. Systemic carnitine deficiency presenting as familial endocardial fibroelastosis: a treatable cardiomyopathy. N. Engl. J. Med. 305 (7), 385–390.
Umetsu, M., Tsumoto, K., Hara, M., Ashish, K., Goda, S., Adschiri, T., Kumagai, I., 2003. How additives influence the refolding of immunoglobulin-folded proteins in a stepwise dialysis system spectroscopic evidence for highly efficient refolding of a single-chain fv fragment. J. Biol. Chem. 278 (11), 8979–8987.
Van de Weert, M., Stella, L., 2011. Fluorescence quenching and ligand binding: a critical discussion of a popular methodology. J. Mol. Struct. 998 (1), 144–150.
Waber, L.J., Valle, D., Neill, C., DiMauro, S., Shug, A., 1982. Carnitine deficiency presenting as familial cardiomyopathy: a treatable defect in carnitine transport. J. Pediatr. 101 (5), 700–705.
Wang, Y., Wang, K., Jette, D.C., Wishart, D.S., 2001. Production of an anti-prostate-specific antigen single-chain antibody fragment from *Pichia pastoris*. Protein Expr. Purif. 23 (3), 419–425.
Wishart, D.S., Knox, C., Guo, A.C., Eisner, R., Young, N., Gautam, B., ... Forsythe, I., 2009. HMDB: a knowledgebase for the human metabolome. Nucleic Acids Res. 37 (suppl 1), D603–D610.
Wu, S., Ke, A., Doudna, J.A., 2007. A fast and efficient procedure to produce scFvs specific for large macromolecular complexes. J. Immunol. Methods 318 (1), 95–101.
Yang, Q., Shi, X., Wang, Y., Wang, W., He, H., Lu, X., Xu, G., 2010. Urinary metabonomic study of lung cancer by a fully automatic hyphenated hydrophilic interaction/RPLC–MS system. J. Sep. Sci. 33 (10), 1495–1503.