



Synthesis, Characterization and Lipophilicity Study of *Brucella abortus*' Immunogenic Peptide Sequence That Can Be Used in the Future Vaccination Studies

Tayfun Acar¹ · Pelin Pelit Arayıcı¹ · Burcu Ucar^{1,2} · Mesut Karahan³ · Zeynep Mustafaeva¹

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Abstract

Brucellosis is primarily a zoonotic disease caused by members of the *Brucella* genus which consists of 11 recognized species based on pathogenicity and host preferences. Peptide vaccines are an alternative strategy to conventional vaccines based on the use of short peptide sequences and engineered to induce highly targeted immune responses, avoiding allergenic and/or reactogenic sequences. In this study, antigenic peptide sequence of *Brucella abortus*, WLAEIKQRSLMVHG, was chemically synthesized by adding tryptophan to the N-terminus of sequence, purified and characterized for the first time in the literature. Molecular weight of the peptide was determined by using LC–MS. A linear response ($R^2 > 0.998$) is observed for peptide in the range of 2–250 $\mu\text{g}/\text{mL}$. The LOD and LOQ values are 0.08 and 0.27 $\mu\text{g}/\text{kg}$ respectively. Precision and accuracy ranges were found to be %RSD < 0.2 and 57.3–103.2%, respectively. Fluorescence property of the peptide was shown via fluorescence spectroscopy measurement. The 3D de novo structure of the peptide was predicted with PEP-FOLD server. According to the data obtained from the lipophilicity study ($\text{LogD}_{7.4} = -3.093 \pm 0.195$), the peptide can not cross the blood–brain barrier if applied via intravenously. This has shown us that peptide can be used as a peptide candidate at the vaccine studies. The production of antigenic peptides as in this study is the main component of the preparation of synthetic vaccine systems.

Keywords Solid phase peptide synthesis (SPPS) · *Brucella abortus* · Peptide vaccine · LC–ESI–MS · Validation · Fluorescence · PEP-FOLD · Lipophilicity

Introduction

Brucellosis is primarily a zoonotic disease caused by members of the genus *Brucella*, which consists of 11 recognized species based on pathogenicity and host preference. There are *Brucella abortus* (cattle), *Brucella canis* (dogs), *Brucella melitensis* (goats or sheep), *Brucella suis* (swine), *Brucella ovis* (rams), *Brucella neotomae* (desert rats) and recently identified strains from marine mammals (De et al. 2008). *Brucella* is considered by the World Health Organization

(WHO), the World Animal Health Organization (OIE) and the United Nations Food and Agriculture Organization (FAO) as the world's one of the most common zoonosis (Abubakar et al. 2012; Li et al. 2018; Roop II and Caswell 2017; Schelling et al. 2003).

Synthetic peptide vaccines represent fragments of protein antigen sequences, which are synthesized from amino acids and assembled into a single molecule or a supramolecular complex or just mechanically mixed; they are recognized by the immune system and induce the immune response (Moisa and Kolesanova 2010; Sesardic 1993). The basis of the new generation synthetic peptide vaccine is the synthesis of peptides that constitute antigenic regions of pathogen spreading. Solid phase peptide synthesis (SPPS) method allows to get larger and more complex peptides synthesized with high purity and yield (Merrifield 1963; Nava-Parada et al. 2007; Oka et al. 2008; Sciutto et al. 2007; Wang et al. 2010). SPPS was originally introduced in the peptide field and there are some reports on the use of microwave irradiation for the preparation of peptides and related species on solid phase

✉ Tayfun Acar
actayfun@gmail.com

¹ Bioengineering Department, Chemistry and Metallurgy Faculty, Yildiz Technical University, Istanbul, Turkey

² Department of Nuclear Medicine and Molecular Imaging, VKF American Hospital, Istanbul, Turkey

³ Department of Bioengineering, Faculty of Engineering and Natural Sciences, Uskudar University, Istanbul, Turkey

(Bacsa et al. 2006; Erdelyi and Gogoll 2002; Matsushita et al. 2005; Yu et al. 1992).

Electrospray ionization mass spectrometry (ESI–MS) is the most important and comprehensive tool for characterization of the synthetic peptides and pure low molecular weight proteins below 30,000 Da. The sensitivity of mass spectrometry is generally excellent for peptides. What make mass analysis of peptides and proteins possible is their ability to enhance non-destructive evaporation / ionisation through ESI. Ionisation occurs by the addition or removal of protons such as [peptide + nH]ⁿ⁺ or [peptide – nH]^{n–}. Since the electrospray has the ability to produce highly charged species, it allows multiple peaks for the same peptide to be calculated from multiple spectra from a single spectrum. Thus, the average of these values can be taken and a more accurate molecular weight can be obtained (Trauger et al. 2002). Very important property of ESI-MS is its ability to directly analyze compounds in aqueous or aqueous/organic solutions, a feature that sets the technique as a suitable mass detector for liquid chromatography (LC). ESI with quadrupole or ion trap analyzers also allows for MS analysis at relatively high LC flow rates (1.0 mL/min) and high mass accuracy ($\pm 0.01\%$), adding a new dimension to the capabilities of LC characterization (Hein 2008; Liu et al. 2011).

In this study, H₂N-WLAEIKQRSLMVHG-COOH antigenic peptide sequence of the *B. abortus*, some of the theoretical properties were given in Fig. 1, was synthesized for the first time in the literature by microwave-assisted solid phase synthesis method (Stevens et al. 1994; Tabatabai and Pugh Jr 1994). Herein, the peptide sequence was synthesized on the basis of the amino acid sequence of *B. abortus* Cu–Zn SOD (superoxide dismutase). SOD might act as a virulence factor by scavenging harmful oxy-radicals as a consequence of phagocytosis by macrophages. Signaling pathways activated by the TLRs (Toll-like receptors) mediated the secretion of IL-12 (interleukin-12) and TNF- α (tumor necrosis

factor- α) by macrophages and mainly by DCs (dendritic cells), in early stages of infection. Whereas in the later stages of infection, the bacterial activity of activated macrophages are mainly due to RNIs (reactive nitrogen intermediates) and ROIs (reactive oxygen intermediates), which are induced by IFN- γ (interferon- γ) produced mainly by Th1 (T helper type) CD4+ cells. The activation of DCs after *B. abortus* infection is characterized by IL-12 and TNF- γ secretion, besides up-regulation in the expression of MHC (major histocompatibility complex) class II. The CD8 + Tc1 (cytotoxic T-cells) kill infected host cells by cytolytic activity (Dorneles et al. 2015; Tabatabai and Pugh Jr 1994).

The purification and characterization of the peptide has also been studied for the first time. The peptide was characterized by using liquid chromatography-electrospray ionization-mass spectrometry (LC–ESI–MS) and fluorescence spectroscopy and purification of the peptide was carried out with a preparative HPLC apparatus. Validation studies were performed for all chromatographic data. The PEP-FOLD online service was used to determine the possible three-dimensional structures of the synthetic peptide. Lipophilicity value (Log D_{7.4}) of the synthesized peptide was determined by shake flask method, wherein, the peptide was partitioned between n-octanol and water phase (octanol/water at pH 7.4) and the concentration of peptide in each phase was determined by RP-LC. *n*-Octanol/water partition coefficient (P) provides direct information on lipophilicity that describes the tendency of distribution of a solute from aqueous phase into biological membranes. The results obtained from this study and the established analytical procedures are the basis for future research and development of synthetic peptide vaccines against the *B. abortus* Disease.

Materials and Methods

Materials

All chemicals used in this study were obtained from commercial sources. Preloaded Fmoc-Gly-Wang LL resin (100–200 mesh), Fmoc-His(Trt)-OH, Fmoc-Val-OH, Fmoc-Met-OH, Fmoc-Leu-OH, Fmoc-Ser(tBu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ile-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Ala-OH, Fmoc-Trp(Boc)-OH amino acids and 1H-Benzotriazolium-1-[bis(dimethylamino)methylene]5-chloro hexafluorophosphate (1-),3-oxide (HCTU), *N*-Hydroxybenzotriazole hydrate (HOBt.H₂O), *N,N*-Diisopropylethylamine (DIEA), *N*-Methyl-2-pyrrolidone (NMP) coupling reagents were purchased from NovaBiochem. *n*-Octanol was bought from Merck. The other chemicals were purchased from Sigma

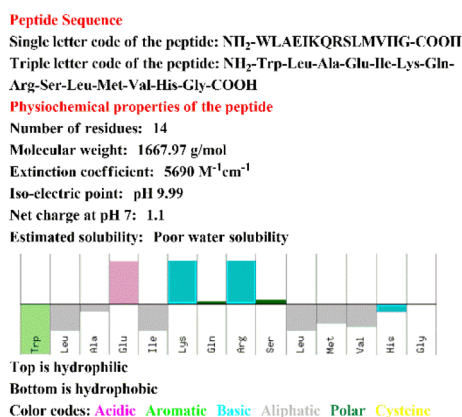


Fig. 1 Some properties of the peptide (Peptide Property Calculator 2017)

Aldrich. Ultra-pure water was obtained from Millipore Milli-Q system.

Methods

Peptide Synthesis

The peptide was synthesized by microwave enhanced solid phase peptide synthesis (SPPS) method in dimethylformamide (DMF) media. The resin beads (370 mg, 0.1 mmol) were transferred to the 30 mL standard glass reaction vessel after swelling in DMF (25 mL). Microwave energy used for the deprotection and coupling steps were respectively 55 W to maximum 45 °C for 2 min and 25 W to maximum 75 °C for 3 min. 20% piperidine solution was used for deprotection process. On the microwave coupling step, HCTU/HOBt. H₂O (0.5 M in 20 mL DMF) and DIEA/NMP (2 M in 15 mL DMF) solutions which used as activators and activator bases were added to fmoc protected aminoacides which dissolved in DMF. These procedures were repeated for all amino acids in the linear peptide sequence. After the coupling of the amino acid residues finished, the peptide bound resin was cleaned by washing with three times in DMF and five times in DCM respectively for preparation of the cleavage process. Then, the peptide was cleaved from the resin using cleavage cocktail prepared with trifluoroacetic acid (TFA)/Thioanisole/1,2-Ethane dithiol (EDT)/Water (90/5/2.5/2.5 (v/v)). Cold diethylether (–20 °C) was used for precipitation. After the centrifugation, precipitated peptide was dried in vacuum (Buchi Glass Oven B-585) (Mustafaeva 2017; Ozdemir et al. 2009; Wang 1973).

Analytical Conditions and Validation Methods

Column and additive choices and defining the appropriate gradient are the main parameters for the best resolution of peptide mixture in liquid chromatography. Frequently C-18 columns are used in peptide separations.

In this work, LC–MS system (Shimadzu LC-MS 2010 EV) with Electro Spray Ionization (ESI) probe was used for characterization of the synthetic peptide. Samples were injected onto Teknokroma Tracer Exel 120 ODS-A 5 µm HPLC column in the dimensions of 20 cm length and 2.1 mm inlet diameter. Elution was gradient from eluent A (water, 0.1% (v/v) TFA) to eluent B (acetonitrile, 0.1% (v/v) TFA) at a flow rate of 2.0 mL/min. The elution programme was as follows: 0–5 min, 20% B; 5–15 min, 20–40% B; 15–30 min, 40–80% B; 30–35 min, 80–20% B. TFA, formic acid, acetic acid and heptafluorobutyric acid are mostly added to the solvent as ion pairing agents for better resolution (Garcia 2005; Shibue et al. 2005). A number of organic solvents can be used in reversed phase HPLC of peptides,

such as acetonitrile, methanol and ethanol (Niessen et al. 2006).

Mass spectrometry of peptides is usually operated in positive ion mode, since separation is performed under acidic conditions. For this reason the ESI was operated in the positive ion mode. The capillary temperature was maintained at 250 °C.

Purification of the peptide was performed by using analytical reversed phase-high performance liquid chromatography (RP-HPLC, SPD-M20A, FRC-10A, LC-8A, CBM-20A, Shimadzu, Tokyo, Japan) equipped with Shim-pack PRC-ODS HPLC column (20 mm × 25 cm) and UV-PDA detector at 210 and 280 nm. Eluent A (water, 0.1% (v/v) TFA) and eluent B (acetonitrile, 0.1% (v/v) TFA) gradient elution was programmed as follows: 0–5 min, 20% B; 5–15 min, 20–40% B; 15–30 min, 40–60% B; 30–35 min, 60–20% B at a flow rate of 14 mL/min. The purified peptide was lyophilized and stored at –40 °C.

The above mentioned LC method was validated in terms of linearity, the limits of detection (LOD) and limits of quantitation (LOQ), intraday and interday precision and accuracy. Standard peptide was accurately weighed and dissolved in distilled water to prepare a stock solution of 1.0 mg/mL concentration. Standard peptide solutions were prepared by dilution with pure water to give seven respective concentrations ranging from 2 to 250 µg/mL. Seven concentrations were injected to the column system, and then the calibration curves were constructed by plotting the peak areas versus the concentrations of each analysis.

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated based on the standard deviation of the noise peak in the chromatogram with using signal-to-noise ratios (S/N) of 3:1 and 10:1, respectively.

The precision of the method was determined by intra-day and inter-day repeatability. The samples at concentrations of 2.5, 10 and 100 µg/mL were given to the column at intra-day and inter-day. The relative standard deviation (RSD, %) was taken as a measure of precision.

The accuracy was evaluated by recovery test. The recovery test was performed by spiking 100 µL of 1, 0.5 and 0.25 mg/mL sample concentration levels to 400 µL 0.05 mg/mL concentration sample, respectively. All samples were then subjected to LC analysis to calculate the recovery rates.

UV–Vis and Fluorescence Spectroscopy Measurements

Absorbance measurements were obtained by using UV–Vis spectrophotometer (UV-1700 pharmaspec, Shimadzu, Japan) within the range of 200–800 nm at room temperature using quartz cuvettes (1 cm path length).

Fluorescence emission spectra peptide was obtained through QM-4/2003 Quanta Master Steady State Spectrofluorometer (Photon Technology International, Canada).

Analysis were run in quanta counting mode with a slit gap of 5 nm for excitation and emission monochromators. Excitation was performed in 270 nm and spectra were measured between 280 and 400 nm's (Filenko et al. 2001).

Pep-Fold Modelling

PEP-FOLD is a very fast online structure prediction service that can be used with high confidence for natural peptides between 9 and 25 aminoacids in aqueous solution as well as, with more caution, in non-aqueous solutions. The 3D structure of the antigenic peptide epitope was predicted using the PEP FOLD (Derman et al. 2014; Shen et al. 2014).

Lipophilicity Assay

It is more convenient to describe the lipophilicity of peptides as the distribution coefficient (log D) because of the containing amino and carboxyl groups, ionizable side chains. The distribution coefficient D, the descriptor of lipophilicity, is a measure of the differential resolution of a compound in two solvent systems that do not interfere with one another. The commonly used solvent system for lipophilicity assay is *n*-octanol/water (Bhal 2007). The water ratio versus the octanol phase, which is assumed the brain membrane, provides strong theoretical data about both whether the synthesized pure peptide surpass the blood brain barrier or not and mechanism of excretion from the body (hepatobiliary or urinary excretion). *n*-Octanol/water (3:1) distribution coefficient ($D_{7,4}$) for the obtained pure peptide sequence was determined by shake-flask method (Leo et al. 1971; Manhold et al. 2008; Sarikaya and Enginar 2012). Reversed-phase high performance liquid chromatography (RP-HPLC) using ACN-water mixture as mobile phase was used to calculate the concentration of the peptide in each phase according to the Eq. 1.

$\text{Log}D_{7,4} = \text{Log}(\text{Distribution Coefficient } \sim (D));$

$$D_{7,4} = \frac{[\text{Peptide}]_{\text{oct}} + [\text{Peptide}^+]_{\text{oct}}}{[\text{Peptide}]_{\text{w}} + [\text{Peptide}^+]_{\text{w}}} \quad (1)$$

Results and Discussion

Characterization of biomolecules like peptides and proteins require the use of several techniques which measure a particular, structural or functional feature. Mass spectrometry is a major method for determining the exact mass of peptides. Liquid chromatography–mass spectrometry (LC–MS) is one of the most frequently used method for the characterization of synthetic peptides. The biomolecules like peptide and protein structure can be examined

by fluorescence spectroscopy through the aromatic amino acids such as phenylalanine, tryptophan and tyrosine found in their structures (Mustafaeva 2017).

The main purpose of this study was to synthesize the previously determined *B. abortus* disease's antigenic peptide sequence, performing chromatographic characterization, identifying the molecular weight and to develop purification procedure of the peptide. The validity of the chromatographic methods has also been studied. Fluorescence property of the peptide was investigated. At the same time three-dimensional structures that the peptide could have were predicted with PEP-FOLD online service. Lipophilicity property of the peptide was investigated via determination of the distribution of peptide in water and octanol by chromatographic method.

Brucella abortus antigenic peptide sequence given in single letter includes WLAEIKQRSLMVHG amino acids was synthesized by solid phase peptide synthesis method under microwave energy. The LC-MS UV chromatogram of the crude peptide was given in Fig. 2 which shows a typical chromatogram of a crude synthetic peptide. It was determined that the peaks seen both 210 and 280 nm on the UV chromatogram at 12.48 min were belong to the peptide.

Synthesized peptide was ionized in MS ionization unit with ESI (+) method and total ion chromatography of peptide was obtained. Total ion chromatogram of the peptide sequence has been showing in Fig. 3. Molecular weight of peptide was calculated from peaks in total ion chromatogram of the peptide. Total ion chromatogram of reverse-phase HPLC of peptide showing the elution of a 1667.60 Da peptide at 12.48 min.

Peptides are typically observed with two to five protons, $(M + 2H)^{2+} - (M + 5H)^{5+}$, in the mass spectrum depending on the mass of the peptide and the number of its basic residues. The *m/z* ratios are typically between *m/z* = 300 and *m/z* = 1500 (Strupat 2005). The mass-to-charge ratios of the ionized ions in the region where the peak appears in the total ion chromatogram between 12 and 13 min in Fig. 3 shown in the mass spectrum of the peptide in

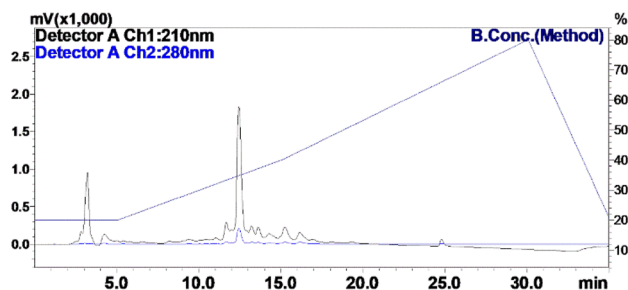


Fig. 2 LC chromatogram of the peptide that demonstrating the elution profile of the crude peptide in 65% purity

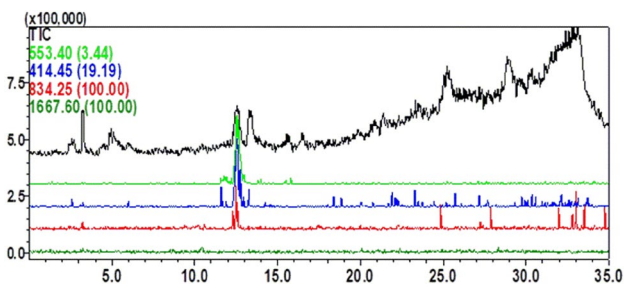


Fig. 3 Total ion chromatogram of the crude peptide. Experimental conditions are the same as described in the text

Fig. 4. Molecular weight of peptide was calculated as follow with using 414.45, 553.40, 834.25 ions. For this peptide the triply charged ions were observed predominantly ($m/z = 553.40$). Some groups such as methyl can be cleaved from small fragments of the peptide formed after ESI process. This can explain that the molecular weight obtained is different from the expected molecular weight (1667.99 Da) (Demarque et al. 2016).

We determined the charge state, z , for each peak and the molecular weight of the peptide using Eqs. (2) and (3) based on the m/z values and peak spacing observed in the charge envelope.

$$z = \frac{(M2 - A)}{M1 - M2} \tag{2}$$

$$MW = \frac{(M1 - A)(M2 - A)}{M1 - M2} \tag{3}$$

Here MW is the molecular weight of the molecule, M1 is the m/z value for the first ion, z is the charge state of the first ion, M2 is the m/z value for a second ion of lower m/z , A is the mass of the adduct ion, that is usually a proton (H^+) where 1.0078 is the atomic weight of H but can be sodium (Na^+) or potassium (K^+) ions from glassware or buffers.

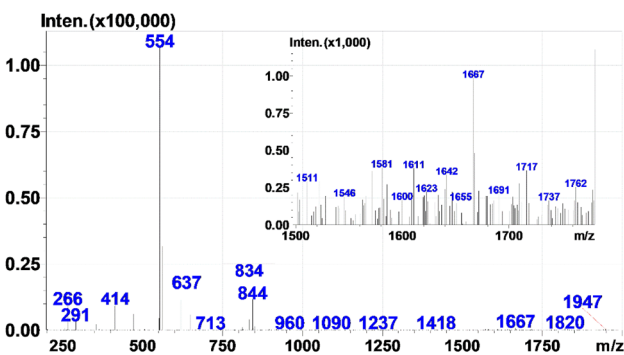


Fig. 4 ESI-MS spectrum of the crude peptide. Intensity given in arbitrary units (AU)

$$z_1 = \frac{(M2 - A)}{M1 - M2} \rightarrow z_1 = \frac{553.40 - 1,0078}{834.25 - 553.40} \rightarrow z_1 = 2$$

$$834.25 = \frac{(MW + z_1H)}{z_1} \rightarrow 834.25z_1 = MW + z_1H \rightarrow MW = z_1(834.25 - 1.0078)$$

$$553.40 = \frac{(MW + z_2H)}{z_2} \rightarrow 553.40z_2 = MW + z_2H \rightarrow MW = z_2(553.40 - 1,0078)$$

$$414.45 = \frac{(MW + z_3H)}{z_3} \rightarrow 414.45z_3 = MW + z_3H \rightarrow MW = z_3(414.45 - 1,0078)$$

$$z_1 = 2; z_2 = 3; z_3 = 4$$

$$[MW + 4H^+]^{4+} = 414.45 \rightarrow MW = 1653.8 \text{ Da}$$

$$[MW + 3H^+]^{3+} = 553.40 \rightarrow MW = 1657.2 \text{ Da}$$

$$[MW + 2H^+]^{2+} = 834.25 \rightarrow MW = 1666.5 \text{ Da}$$

$$MW_{\text{observed}} = 1659.20 \text{ Da } MW_{\text{theoretical}} = 1667,99 \text{ Da}$$

RP-HPLC elution profile illustrating the purification of the synthetic peptide in Fig. 5. All the peaks scanned in turquoise color were collected in fractions (FR1–FR9) and each were re-analyzed in the LC-MS instrument to confirm the molecular weight. 414.45, 553.40, 834.25 different protonated ions of the synthesized peptide were detected in the electrospray ionization mass spectrum of FR5 (red peak in Fig. 5 at 24.2 min) as shown in the Fig. 6. We could not have any significant result for other fractionated peaks (FR1–FR9 except FR5) from mass spectrum (data not shown). It is believed that these peaks are caused by the change of the

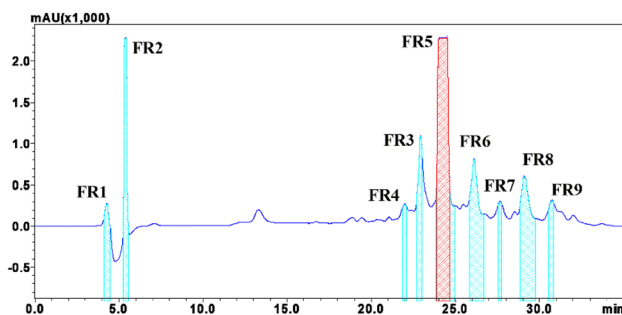


Fig. 5 Preparative HPLC chromatogram of the crude peptide. Experimental conditions are the same as described in the text

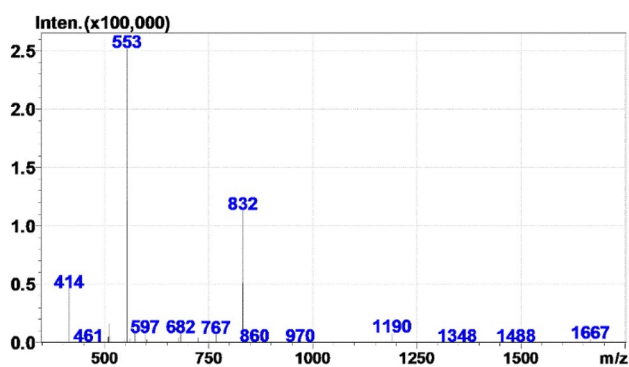


Fig. 6 ESI-MS spectrum of the purified peptide. Intensity given in arbitrary units (AU)

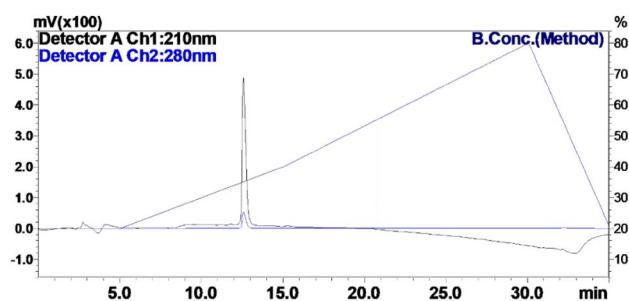


Fig. 7 LC UV chromatogram of the purified peptide in 98% purity. Experimental conditions are the same as described in the text

mobile phase concentration or caused by the by-products in the solid phase peptide synthesis. The LC-UV chromatogram of the purified peptide after confirming the molecular weight by ESI-MS was also given in the Fig. 7. 98% pure peptide was obtained.

Linearity was evaluated by analysing seven different concentrations of standard solutions. The calibration curve was obtained in a concentration range from 2 to 250 $\mu\text{g}/\text{mL}$ and found to be linear for peptide with coefficient of determination greater than 0.998. The LOD and LOQ values for the

chromatographic analyses were found 0.08 and 0.27 $\mu\text{g}/\text{kg}$, respectively. The precision 0.68 and 0.045% for intra-day, and 5.61 and 3.95% for inter-day, respectively. The accuracy values obtained in the recovery tests were between 57.63 and 103.20%. The validation study of the chromatographic method has shown that the method was acceptable in terms of linearity, accuracy and precision.

Biomolecules like peptide and protein characteristically absorb light maximally at 280 nm through the aromatic amino acids found in their structure. It has been identified that the peptide was absorbed light at 280 nm (Fig. 8a).

The fluorescence properties of the tryptophan (Trp) in peptides provide a sensitive and informative data for many different types of studies of synthetic peptides. In our studies of bioconjugation of synthetic peptides with biopolymers, we are able to do the fluorescence studies when Trp residues impart fluorescence properties to the polymer peptide conjugates. Therefore, when the peptide does not contain a Trp, it is often useful to add one. In our present work Trp was added to the N-terminus of the peptide sequence for spectrofluorometric analysis (Alston et al. 2008; Mansuroglu and Mustafaeva 2012; Mustafaev et al. 2004, 2006). It is understood from the high fluorescence intensity value in the fluorescence spectrum given in Fig. 8a that the peptide has fluorescence property due to the Trp amino acid at the N-terminal of the peptide.

PEP-FOLD only attempts to provide the structure of lowest effective energy. PEP-FOLD is available as free server and currently limited to the study of peptides with 9–37 aminoacids. In the present work, de novo three-dimensional models of the 12 amino acid peptide sequence were illustrated in Fig. 8b which was predicted by PEP-FOLD.

Lipophilicity analyze gives information about the passage or not of a substance through the blood-brain barrier and mechanism of excretion of the substance from the body. According to the lipophilicity study $\text{LogD}_{7.4}$ value was found -3.093 ± 0.195 . Strongly negative value of $\text{LogD}_{7.4}$ gives us to draw conclusion that the peptide could be applied as

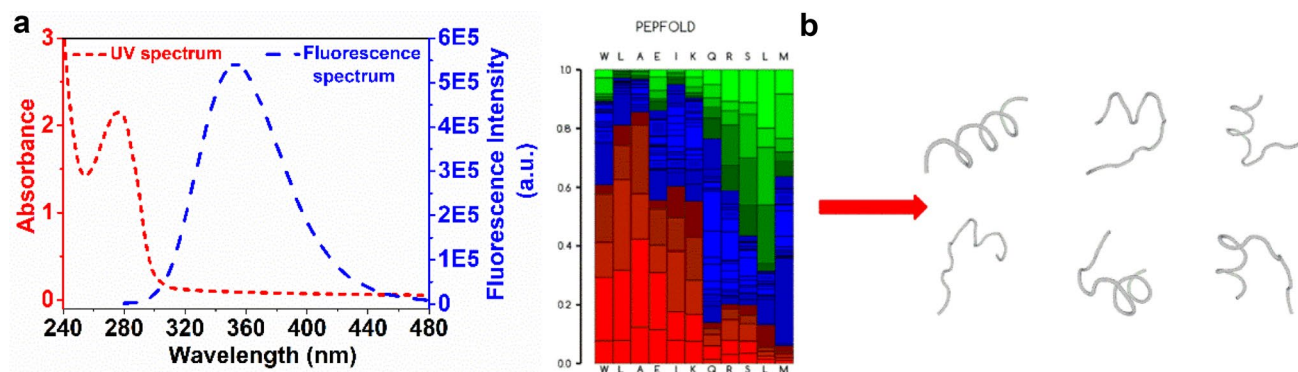


Fig. 8 a Absorbance and fluorescence spectrum of the peptide, b 3D de novo model of the peptide

a strong antigen candidate that not to cross the blood–brain barrier.

These results will be a milestone for the basis for further development of the synthetic peptide vaccine and planning of future confirmatory trials.

Conclusion

In the paper, the *Brucella abortus* disease's one of the most antigenic synthetic peptides was synthesized by microwave-assisted solid-phase peptide synthesis method and purified with preparative HPLC. The purified peptide was characterized with LS–ESI–MS, fluorescence spectroscopy and PEP-FOLD server. The validation study of the chromatographic method has shown that the method is acceptable in terms of linearity, accuracy, precision and reproducibility. According to the negative lipophilicity value, the synthetic peptide has shown that it may be an antigen that can be used in vaccination studies. In this study, we performed synthesis and characterization of the peptide which has been proven in the literature that it has antigenic property that can be used in the future studies in order to produce synthetic vaccine formulations against *Brucella abortus* disease.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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