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Designing and overproducing a tandem epitope of gp350/220 that shows a potential to become an EBV vaccine

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Abstract

Background: Epstein-Barr virus (EBV) can cause cancer in people from around the world. There is no EBV vaccine available for use on a global scale. However, emerging evidence suggests that the epitope on the gp350/220 capsid protein may be developed into an EBV vaccine. Nevertheless, the production of small, single epitope is challenging of stability issues and possible alteration of peptide structure. In this study, a tandem epitope was developed consisting of three single epitopes, aimed to improve stability, antigenicity and preserve epitope structure.

Materials and methods: A tandem epitope was designed using bioinformatics based on the epitope structure of the gp350/220 protein. The tandem epitope structure was analyzed using a protein folding method with Abalone software, which was further refined via YASARA force field and molecular repairing using a FoldX method. Immunogenicity was examined with Epitopia software, whereas allergen properties were tested using AlgPred. The pattern of the tandem epitope binding with anti-gp350/220 antibodies was performed using Z-dock and snugDock. The tandem epitope was then overproduced in *E. coli* strain BL21 as a host cell.

Result: Our model demonstrated a successfully designed and overproduced tandem epitope. The tandem epitope demonstrated a similar structure compared with the

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epitope of whole protein gp350/220. Our epitope also demonstrated non-allergen and antigenicity properties, and possessed antibody binding patterns consistent with whole protein gp350/220.

Conclusion and recommendation: These data suggest a novel tandem epitope composed of three similar epitopes demonstrates antigenicity, structure, and binding properties consistent with whole protein gp350/220. We also demonstrate successful production of the tandem epitope using *E. coli* strain BL21 as a host. Future *in vivo* experimental animal research is necessary to test the ability of this tandem epitope to stimulate antibody production.

Keywords: Vaccines, Biotechnology

1. Introduction

Epstein-Barr virus (EBV) can cause epithelial and lymphoid malignancies [1], which account for 1.8% of all cancer related deaths [2]. While EBV has been observed in approximately 90% of stomach cancer cases in individuals living in Portugal [3], this virus is also known to occur worldwide affecting people in Southeast Asia, Central Africa, Mediterranean Africa and the United States [1]. While it has been demonstrated that one way to prevent the spread of EBV is through vaccination [4], to date, an EBV vaccine has not been developed that can be readily used on a worldwide scale [4]. Current EBV vaccine development strategies have focused on using gp350, which is a protein present in high abundance on cell surfaces [4]. As such, because it has been proposed the anti-gp350/220 monoclonal antibodies may be able to neutralize EBV [5], further development of a vaccine using this protein is warranted. In particular, it is suggested that the gp350/220 protein functions by binding host receptor CD21, and thereby mediates human cell internalization [4]. The binding of antigenic epitopes to antibodies or B-cell receptors is needed for stimulating B-cell activity, B-cell proliferation and antibody production [6]. Therefore, the exact nature of this antigenicity is essential and is one of the primary features that should be considered when designing a vaccine [7].

Although most approaches used for EBV vaccine development are based on the whole gp350/220 [4], it has also been demonstrated that a small peptide application (i.e., epitope) can bind antibodies and neutralize antibody binding with gp350/220 [5]. This suggests that development of vaccines based on a single epitope is promising for this line of study. Accordingly, development of an EBV vaccine based on the gp350/220 conserve epitope may be beneficial in protecting against a broad spectrum of EBV strains while demonstrating high levels of safety for humans. The EBV envelope gp350/220 protein has 907 amino acid residues that has 21 sequence variants or polymorphic [8]. Identification of additional potentially effective gp350/220 epitopes can be performed using bioinformatics [5, 9, 10] since open availability of

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the EBV sequence database permits the ability of researchers to closely study and map the conservative region of the epitope [10]. Indeed, our group previously identified a single epitope on the gp350/220 capsid protein using the bioinformatics approach [8]. We observed that epitope of the single gp350/220 capsid protein epitope is able to stimulate IgG production *in vitro* [11].

Despite the therapeutic possibilities, production of peptides such as the epitope continues to be a challenge [12]. This is largely because overproduction of a small peptide on host bacteria, yeast and mammalian cells is a complicated process because small peptides tend to have low stability and creates aggregates that are toxic to the host [13, 14]. As such, based on previous work from our lab (unpublished), overproduction of single epitopes does not work using *E. coli* as the host cell. Following failure of the single epitope model, our group developed a tandem epitope consisting of three single epitopes. The joining of three single epitopes into a single tandem epitope is suggested to enhance epitope stability and preserve epitope structure and antigenicity properties.

Consistent with the suggestion that a tandem epitope will increase protein stability, which makes it easier to produce in cell culture, we successfully demonstrated in this study the design and overproduction of a tandem epitope. We predicted that our tandem epitopes retained antigenicity, structure and binding properties with respect to anti-gp350/220 antibodies. Lastly, because sensitivity of tandem epitopes has been proposed to increase resulting in heightened immune responses [15], further research is needed to test the ability of our novel tandem epitope in being able to stimulate antibody growth via *in vivo* experiments conducted in animal models.

2. Materials and methods

2.1. Modeling epitope structure

Single and tandem epitope structures were modeled using the protein folding method in Abalone software [16]. The results of this model were tested for validity using the Ramachandran plot in the Discovery Studio software [17]. The structure of modeling results was further refined in two stages, 1) performing energy minimization using the YASARA force field [18], and 2) molecular repairing using FoldX [19]. Following refinement at each stage, a validity check was carried out using a Ramachandran plot, and the stability of protein energy was calculated. The suitability of the epitope structure model is based on its relevance using the Ramachandran plot.

2.2. Analysis of allergic responses and immunogenicity potential *in silico*

Our modeled epitope was analyzed for immunogenicity properties using Epitopia software, which is a webserver available for predicting B-cell epitopes [20]. This

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analysis program compared immunogenicity properties across the single epitope, tandem epitope and the whole protein gp350/220. Compared with the whole protein gp350/220, it was anticipated that the tandem epitope would demonstrate no decrease in antigenicity. In addition, the tandem epitope was analyzed for its potential to elicit an allergic immune response. In predicting whether the tandem epitope was a potential allergen we used AlgPred software, which can be used to predict allergenic proteins and also map IgE epitopes [21].

2.3. Prediction of binding patterns and affinity

Binding patterns and affinity predictions of tandem epitopes with respect to anti-gp350/220 antibodies were performed using molecular docking. This bond analysis was tested through two stages. The first stage included blind docking between ligand (antibody) and receptor (tandem epitope and whole protein gp350/220) via Z-dock software [22]. Resulting docking data were then adjusted according to binding of antigens via snugDock software [23]. Following, bonding patterns between tandem epitopes and antibodies were then compared to bonding patterns between whole protein gp350/220 and antibodies. These bonding patterns are expected to demonstrate whether an epitope tandem can bind in the same way as whole protein gp350/220. Lastly, the structure of anti-gp350 antibody was modeled using a homology modeling server for the antibody variable region of interest (RosettaAntibody) [24] according to mAb 72A1 heavy- and light-chain variable region sequences [5].

2.4. Protein stability prediction

Prediction of protein stability of single and tandem epitopes was calculated using ProtParam software [25]. This analysis is essential for identifying physicochemical properties and epitope half-life in various cell types. The physicochemical properties analyzed included molecular weight, theoretical pI, instability index, aliphatic index and grand average of hydropathicity. The half-life was predicted in mammalian, yeast and *E. coli* cells as these cells can be utilized as hosts for production of recombinant proteins.

2.5. Transformation and recombinant protein overexpression

The sequence encoding the tandem epitope was synthesized by Genescript and inserted into pMAL-p5x plasmid. The sequence was inserted in the downstream of Maltose binding Protein (MBP), and just between Factor Xa in upstream and rrnB T1 terminator sequence. The insertion results were checked for validity based on the excision of the restriction enzyme (AflIII; NEB Cat No. R0541S). Following, the *E. coli* strain BL21 was transformed using a newly constructed plasmid. Briefly,

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the transformation was performed by mixing 1 ul (50 ng) of Plasmid with 25 μl competent cell, and incubate on ice for 30 min, the heat to 42 °C for 90 seconds, and then put on ice for 5 minutes [26]. The transformed cell was added 0.1 ml SOB and incubate at 37 °C for 2 hours. Next, selection on the successful transformation was performed by growing *E. coli* transformant on LB media enriched with ampicillin 100 ug/ml for overnight at 37 °C. Overproducing of the recombinant protein was performed as follow, transformant cells were then grown in LB broth, which was enriched with glucose and ampicillin. After cell cultures had reached OD 0.5−0.7, they were induced with IPTG 0.3 mM and incubated for 2 hours at 37 °C at 150 rpm. The protein was isolated according to the method of NEB [27], the 50 ml cell suspense was withdraw and centrifuged at 4500 rpm for 20 min, pellet cell was resuspended with 20 ml first buffer 30 mM Tris (pH 8), 20 % sucrose, and 1 mM EDTA (pH 8). The cell suspense was shake for 10 minutes at room temperature, then centrifuged for 8.000 g for 20 minutes at 4 °C. The pellet was resuspended with 20 ml $MgSO_4$ 5 mM cold, and then shake in cool temperature for 10 min. The sample ten was centrifuged at 8.000 g for 20 minutes at 4 °C. The Supernatant was added 400 ul Tris-Cl 1 M (pH 7,4). Measurement of protein concentration was performed using the Bradford method, and molecular weight on SDS was determined by measuring the distance of Rf at 10% acrylamide gel concentration. Crude protein was then purified using MBPTap HP 1ml column chromatography (GE Healthcare, cat. 28-9187-79).

2.6. Examination binding activity between tandem epitope and anti-gp350/220

The pMAL-p5X vector has the signal peptide on pre-MBP directs fusion proteins to the periplasm; for fusion proteins that can be successfully exported to the periplasm of *E. coli*. Sixteen microliter of periplasmic protein (2.4 mg/ml) or gp350/220 protein (1 mg/ml) (MyBioSourceeach) was diluted with 80 ul of coating buffer, put on 96 well plate and incubated overnight at 4 °C. The solution was discarded and washed with PBST for 5 minutes three separate times. For each well, we added 200 μL blocking buffer (0.25% gelatin in PBST 0.2%) for 1 hour at room temperature. The solution was discharged and washed with PBST for 5 minutes three separate times. Rabbit polyclonal anti-gp350 (Catalog number: 142971, NovoPro) diluted with PBST (1:5000) was added to wells, and incubated for 1 hour at room temperature. The solution was then discharged and washed with PBST for 5 minutes three separate times. HRP Donkey anti-rabbit IgG (Catalog number: 406401, BioLegend) diluted with PBST (1:80.000) was added to wells, and incubated for 1 hour at room temperature. After washing three separate times, 100 ul TMB High Sensitivity Substrate Solution (Catalog number: 421501, BioLegend) was added to wells for 2 min, followed by the addition of 50 ul of stop solution. The absorbance was read at 450 nm via ELISA Reader ELx 808 BioTek.

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3. Results

Our previous research identified a single epitope by using bioinformatics [8] that able stimulate IgG production *in vitro* [11], which is indicated the epitope has antigenic properties. As a subsequent step to this, overproduction of these proteins was attempted. However, producing a single epitope, which is a small epitope inside a cell, has its own challenge. Thus, a tandem epitope composed of three same epitopes or three single epitopes linked to the amino acid Methionine was developed – this is referred to as a tandem epitope. Our modeling data demonstrate the epitope structure of single and tandem epitopes to be similar with the epitope associated with the whole protein gp350/220 (Fig. 1).

Modeling of the epitope was carried out using *ab inito* modeling based on protein folding modeling using Abalone software. The model structure of the Abalone was then evaluated for its energy protein stability. This was then refined using YASARA software. Refinement was performed by conducting an energy minimization using the YASARA force field [18], and molecular repairing using foldX [19]. Refinement results demonstrated that protein stability had the smallest energy values equal to 18.32 kcal/mol and 63.45 kcal/mol for the single and tandem epitope, respectively (Table 1).

Furthermore, the antigenicity of the epitope in whole protein gp350/220 was compared with the single and tandem epitopes using Epitopia software; a webserver for predicting B-cell epitopes [20]. The results of this analysis demonstrate that antigenicity profiles of single and tandem epitopes are similar in comparison to the epitope in the whole protein gp350 (Fig. 2). Analysis of these epitopes progressed to the establishment of their allergenic properties. To develop the vaccine, the antigen used should not be an allergen. The prediction of the epitope potential as an

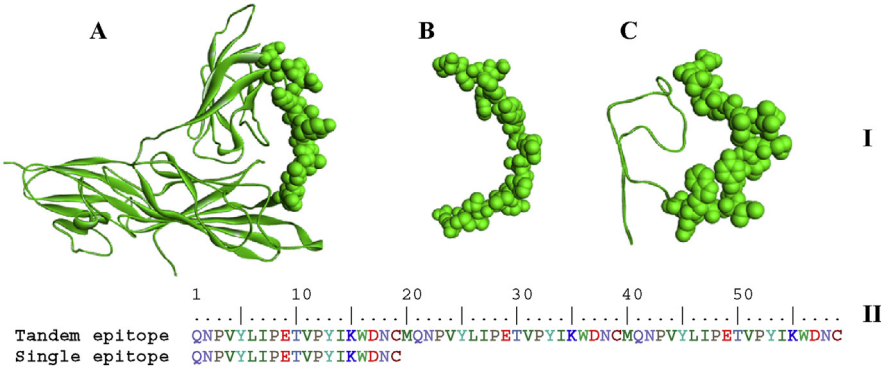

Fig. 1. The structure of whole protein gp350/220 from EBV. The epitope on whole protein gp350 (A) has a similar structure with the single epitope (B) and tandem epitope (C). An epitope structure visualized in the form of a space-filling calotte model, CPK (I). Comparison of tandem epitope sequences (joining of three single epitopes) and a single epitope are presented in panel II.

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Table 1. Protein stability of molecules before and after preparation.

Preparation	Protein stability (kcal/mol)	
	Single epitope	Tandem epitope
Original	33	163
Energy minimization	24	82
Protein repair	18	63

allergen was performed using AlgPred, a prediction of allergenic proteins and mapping of IgE epitopes [21]. We found that the tandem epitope has no IgE epitope and is a non-allergen (Table 2). Likewise, we also observed that the tandem epitope demonstrates the same antigenicity as the epitope of the whole protein gp350/220; and that it does not show non-allergen properties or trigger the stimulation of IgE production.

Furthermore, the tandem epitope was tested for binding to anti-gp350/220 antibodies *in silico*. This bonding analysis was performed using docking proteins in two stages. The first stage was blind docking between ligand (antibody) with the receptor (tandem epitope and whole protein gp350/220) employing Z-dock software [22], The docking result was then adjusted for the binding according to antigen-antibody binding using snugDock [23]. The results of docking analysis demonstrated that the tandem epitope and whole protein gp350/220 have bound with the similar amino acid of

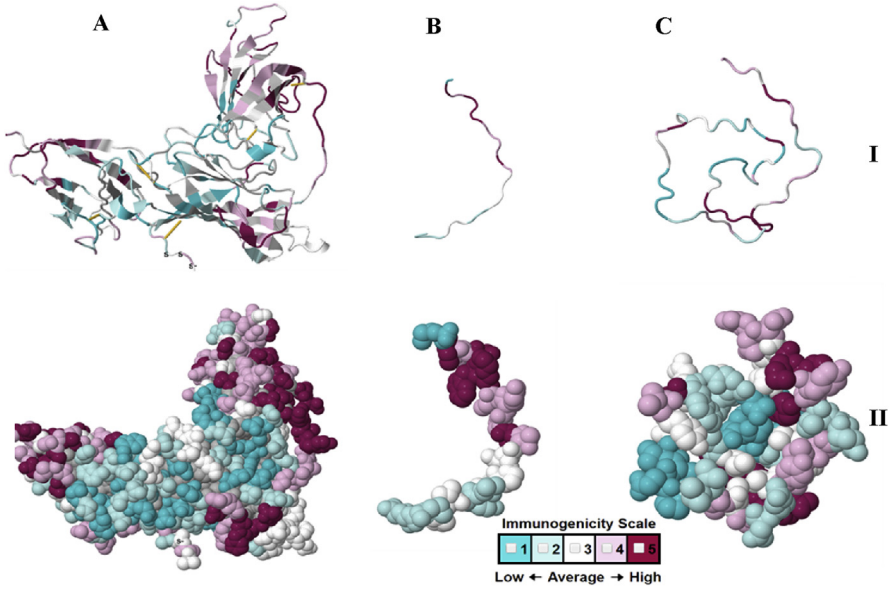

Fig. 2. Immunogenicity scale of gp350/220 epitope of EBV. Epitope in protein gp350/220 (A) demonstrates similar immunogenicity compared with single (B) and tandem epitopes (C). The map of immunogenicity is visualized in the form of a cartoon structure (I), and in the shape of a space-filling calotte model, CPK (II).

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Table 2. Prediction of allergen potential of the tandem epitope.

NO	Analysis	Result
1	Prediction by mapping of IgE epitope	None
2	Allergen prediction by amino acid composition	Non-allergen
3	Prediction based on di-epitope composition	Non allergen

antibody (Fig. 3 and Table 3). This pattern indicates that the tandem epitope has a similar function with the whole protein gp350/220 in binding to the antibody.

The tandem epitope was analyzed using calculated physical properties to determine suitable production cell type. This analysis was carried out using *in silico* method by employing ProtParam software [25]. The results of the bioinformatics prediction suggested that the tandem epitope has a stable half-life in *E. coli*, which may suitable for a host for overexpression of the tandem epitope. The half-life of proteins in the host is predicted according to the 'N-end rule'. the N-terminal amino acid, simply determines the half-life of proteins in the different of the host [28, 29, 30]. The epitope has Gln (Q) in the N-end terminal that estimated has longest half -life in

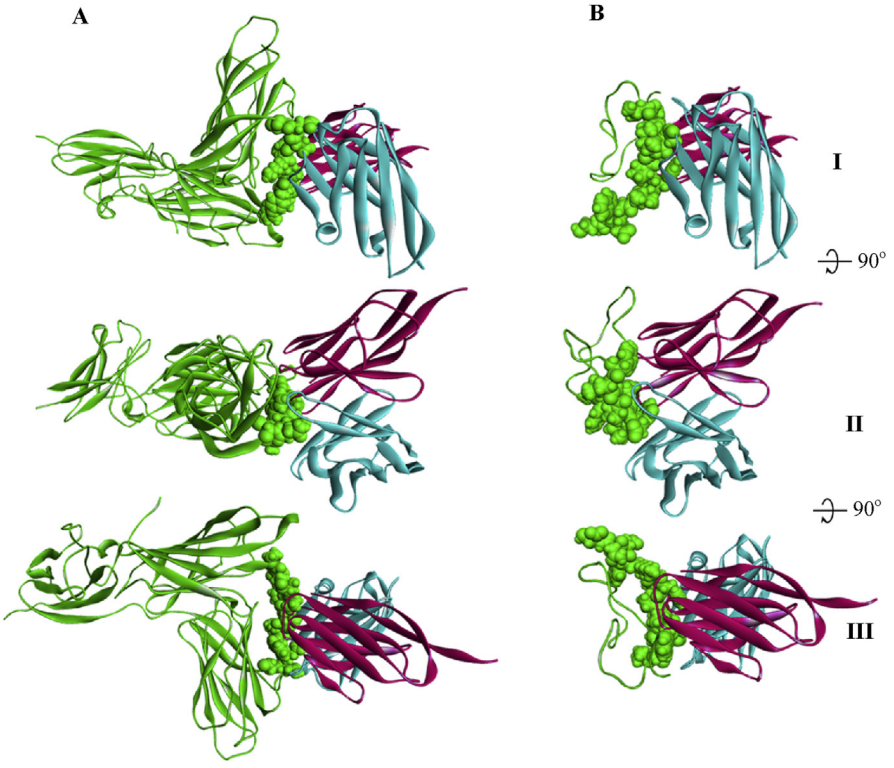

Fig. 3. Epitope binding pattern with anti-gp350/220 antibody. The antibody has the same binding pattern both on gp350/220 (A) and the tandem epitope (B). The H-variable chain (cyan) and L-variable chain (magenta) bind to the same epitope position (I), although its position is rotated 90° (II) and 180° (III).

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Table 3. The binding profile between antibody with tandem epitope or native protein.

	Complex Protein Stability (kcal/mol)	Interaction Energy (kcal/mol)		Interface Residues	
		H-variable	L-variable	H-variable	L-variable
Tandem epitope	608.88	67.21	238.38	**E35**; W47; **Y50**; S52; S52; **T56**; **E58**; D61; K64; **W95**; **N97**; **Y98**; **P99**; **Y100**	**L2**; **E27**; **N28**; **V30**; **T31**; **Y32**; **Y49**; A51; **S52**; **N53**; S65; S67; **A68**; **T69**; F71; **Q90**; **Y92**; **S93**; **Y94**; **P95**; **Y96**
Native protein	957.51	123.46	141.18	S31; F32; **E35**; **Y50**; **T56**; **E58**; R94; **W95**; **N97**; **Y98**; **P99**; **Y100**	**L2**; S26; **E27**; **N28**; **V30**; **T31**; **Y32**; **Y49**; **S52**; **N53**; **A68**; **T69**; **Q90**; **Y92**; **S93**; **Y94**; **P95**; **Y96**; T97

the *E. coli* (Table 4). The molecular weight of tandem epitope is higher than three time of single epitope caused by Methionine was inserted between the epitopes (Fig. 1).

Following this, the sequence encoding the epitope was inserted into the pMAL-p5x plasmid. The *E. coli* strain BL21 was transformed using a newly constructed plasmid, which was then over-expressed. The result of this study is the recombinant protein of the tandem epitope-MBP with a molecular weight of 50 kDa. The tandem epitope able to bind with gp350/220 antibody by ELISA (Fig. 4).

4. Discussion

For the first time, this study designed a functional tandem epitope consisting of three same/single epitopes. These data suggest that this epitope is able to maintain a

Table 4. Physicochemical characteristics and stability in the host.

Characters	Singe epitope	Tandem epitope
Molecular Weight	2,292.6 Da	7,104.26 Da
Theoretical pI	4.37	4.30
The estimated half-life in mammals	0.8 hours	0.8 hours
The estimated half-life in yeast	0.6 hours	0.6 hours
The estimated half-life in *E. coli*	10 hours	10 hours
Instability index	25.91	34.21
Aliphatic index	92.11	88.98
Grand average of hydropathicity	−0.353	−0.276

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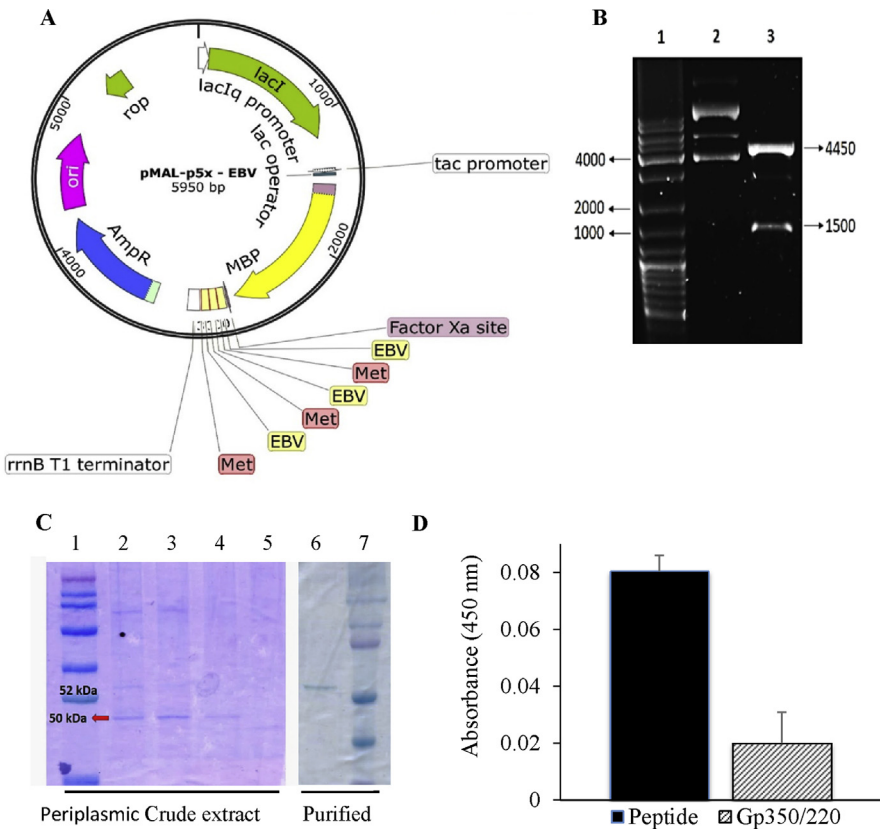

Fig. 4. Overproduction of epitope in *E. coli*. The sequence of the tandem epitope is inserted between MBP and rrnB T1 terminators on plasmid pMAL-p5x (A). The accuracy of the tandem epitope sequence in the plasmid was analyzed using the pattern of cutting by AflIII enzyme restriction; DNA ladder (1), uncut plasmid (2), and cut plasmid (3) (B). Overexpression of tandem epitope using *E. coli* BL21 (C), both induced with IPTG (line 2 and 3), without induction (line 4) compared to the control (line 5); purified protein using maltose column (6) and protein marker (7). The epitope from crude extract was able to bind with polyclonal antibody that is comparable to Gp350/220 protein, as positive control (D).

structure that is similar to the epitope of whole protein gp350/220. Moreover, results of our affinity binding analysis illustrate that our epitope can bind with anti-gp350/220 antibody in the same position as whole protein gp350/220. These observations have immediate implications as the introduction of an epitope with an antibody or B-cell receptor is essential to boost B-cell activity to proliferate and produce antibodies [6].

Results from our antigenicity analysis demonstrate that the tandem epitope is antigenic, and shows a similar degree of antigenicity compared with the epitope associated with whole protein gp350/220. As such, the nature of this antigenicity is crucial and acts as one of the conditions that should be present when designing a vaccine [7]. In addition to antigenicity, the main requirement of a vaccine is that it does not induce an allergic response. Accordingly, these data suggest our novel tandem epitope is not an allergen and has no epitope for IgE.

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The recombinant tandem epitope produced in *E. coli* strain BL21 succeeded in obtaining a 50 kDa protein recombinant of tandem epitope pMAL. Overproduction of a small epitope often requires a well-planned strategy because small epitopes tend to be unstable, or form aggregates inside the cell making it difficult to extract from the cell. Our previous research illustrates such a case, which provides a rationale for testing the tandem epitope. The present observations are consistent with others demonstrating that the tandem epitope method improves the ability to stimulate the immune system [31, 32, 33].

5. Conclusion

A tandem epitope consisting of three single epitopes is suggested to demonstrate antigenicity similar to that of the epitope of whole protein gp350/220. Moreover, these data suggest that our novel epitope is predicted to be capable binding antibodies on the same side demonstrated by the gp350/220 protein. Finally, we demonstrated successful production of the tandem epitope using *E. coli* strain BL21 as a host cell. Additional research in animal models are needed to confirm activity and to induce antibody production.

Declarations

Author contribution statement

Widodo, Muhaimin Rifa'i: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Nadya Veronica Margarecaesha Anyndita, Nurul Dluha, Karimatul Himmah, Mulya Dwi Wahyuningsih: Designed plasmid, Performed the experiments; Analyzed and interpreted the data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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