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Improved fusion tag cleavage strategies in the downstream processing of self-assembling virus-like particle vaccines

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ABSTRACT

Virus-like particle (VLP) vaccines are emerging as an exciting platform for the delivery of antigenic modules to enable engineering of desirable immunological outcomes, both in therapeutic and prophylactic settings. The processing of these macromolecular assemblies raises new challenges not previously encountered for simpler protein therapeutics. Microbial expression of viral precursor protein complexes (capsomeres) and their subsequent cell-free self-assembly represent a new and technologically interesting pathway to VLP vaccines, yet significant scientific and engineering challenges remain. Among these, the use of thrombin within the existing laboratory process must be eliminated, for regulatory, cost and product quality reasons. Here, the use of alternatives to thrombin is explored. It is shown that tobacco etch virus protease (TEVp) is a viable alternative to thrombin, and leads to higher-quality VLP product; as TEVp has no known human physiological or biochemical role, its bioprocessing acceptability is expected to be higher than for thrombin. The results presented here enhance the scale-up potential of microbial VLP processing, and suggest that TEVp may be a preferable enzyme for use in other bioprocess settings, including those related to the processing of less complex biologics.

Keywords: Virus-like particles; Thrombin; TEVp; Enzyme; Murine polyomavirus; GST

1. Introduction

The success of VLP-based vaccines for human papillomavirus (Paavonen et al., 2009; Villa et al., 2005) and hepatitis B (McAleer et al., 1984) demonstrates the potential of virus-like particles (VLPs) as safe and efficacious vaccines. Research continues into a number of VLP-based vaccines including influenza (Krammer and Grabherr, 2010; Quan et al., 2010), HIV (Young et al., 2006), norovirus (Herbst-Kralovetz et al., 2010), and rotavirus (Bertolotti-Ciarlet et al., 2003; Ciarlet et al., 1998). Increasingly, research is directed to engineering the surface of modular VLPs to enhance immunogenicity of selected antigens (Chackerian et al., 2001; Jennings and Bachmann, 2008; Middelberg et al., 2011; Slupetzky et al., 2001; Wibowo et al., 2012). A major challenge to the translation of VLP vaccine research into effective vaccine products is the development of faster and more cost-effective bioprocesses that deliver VLP products at the required industrial scale (Buckland, 2005; Pattenden et al., 2005), a challenge common to most protein therapeutics (Nfor et al., 2009). Microbial expression technology is being explored to produce the major structural subunit proteins of a number of different VLPs (Arora et al., 2012; Chen et al., 2005; Li et al., 2012; Marcekova et al., 2009; Middelberg et al., 2011; Wada et al., 2012), and may address the need for a cheaper and more rapid VLP manufacturing process.

A low-cost, rapid-response vaccine approach combining the advantages of a VLP subunit platform and microbial manufacturing processes to target influenza and Group A Streptococcus was recently reported (Chuan et al., 2013; Middelberg et al., 2011; Rivera-Hernandez et al., 2013; Wibowo et al., 2012). With this synthetic biology approach (Foo et al., 2012; Rollié et al., 2012), an antigenic module is genetically fused to the murine polyomavirus (MuPyV) subunit structural protein VP1, resulting in modular VP1 that forms capsomeres

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when recombinantly expressed in *Escherichia coli* (*E. coli*). The capsomeres are purified and assembled, in cell-free downstream processes, into modular VLPs (MuPyV comprises 72 pentamers/capsomeres of VP1, or 360 copies of VP1). The expression of the 42.5 kDa major structural protein MuPyV VP1 in *E. coli* has been optimised, with fusion to a 26 kDa glutathione S-transferase (GST) tag for downstream processing efficiency (Chuan et al., 2008b). With the reported approach, the GST-tag is cleaved from the MuPyV VP1 protein during downstream processing using thrombin enzyme, allowing subsequent chromatographic separation to yield capsomeres that are self-assembled into VLPs (Middelberg et al., 2011). The use of GST as a fusion tag, enzymatically cleaved by thrombin, is not exclusive to MuPyV VLP production, and has been reported for human papillomavirus VLP L1 coat protein also (Hanslip et al., 2006, 2008). This research will focus on this proteolytic cleavage step in the purification of MuPyV VP1 protein, for *in vitro* self-assembly of VLPs.

Most of the pGEX GST fusion vectors (GE Healthcare), as used to express MuPyV VP1 in *E. coli* (pGEX-4T-1) (Garcea et al., 1987; Middelberg et al., 2011), use thrombin or thrombin-like proteases (FactorXa and PreScission™ protease) as the proteolytic tag-separation enzyme. However, thrombin is the main initiator of the coagulation cascade upon human injury (Bode, 2006) and has been used in a surgical setting for over 60 years as an accelerant for blood coagulation (Ofosu et al., 2009). Complete removal of thrombin from therapeutic products would be required for approval from any therapeutic regulatory body, and although removal may be possible with further chromatography steps, this adds to the processing costs and becomes a manufacturing bottleneck to the downstream processing (Nfor et al., 2009). Validating the removal of this enzyme within a regulated product setting will be difficult, necessitating high levels of process redundancy, with additional unit operations included only to ensure process robustness and operability with regard to thrombin removal. Furthermore, there is no convenient procedure for the expression and purification of recombinant thrombin at bench-level scale (Waugh, 2011). Commercially sourced thrombin is costly even at laboratory scale (Sigma–Aldrich Thrombin T6634: >$500), thus adding to downstream processing costs.

Several studies on thrombin have shown that while cleavage is reasonably specific for the common sequence motif [LVPR↓GS] (Waugh, 2011), specificity is not absolute and can occur at other non-specific sites within a protein (Chang, 1985; Jenny et al., 2003), resulting in product heterogeneity. Non-specific proteolysis of MuPyV VP1 has been previously observed (Liew et al., 2010; Wibowo et al., 2012), resulting in product heterogeneity of a therapeutic protein. This heterogeneity necessitates additional downstream processing to obtain homogenous end product (Fee, 2003). Thus the use of thrombin for the proteolytic enzyme to release the fusion tag may increase complexity in the downstream processing of VLPs as vaccines. A preferable strategy would involve selection of a proteolytic enzyme not involved in human physiological and biochemical networks, thus facilitating regulatory approval, and one that can be cost-effectively produced. Two enzymes meeting these criteria that are already proven in fusion tag removal processes, though not in relation to VLP downstream processing, are enterokinase (Collins-Racie et al., 1995) and tobacco etch virus protease (TEVp) (Cabrita et al., 2007). In this research we therefore explore the use of thrombin, enterokinase and TEVp for the cleavage of the GST-VP1 fusion protein for purification of MuPyV capsomeres.

In this study, bioinformatics was first used to identify potential enzyme cleavage sites within the GST-VP1 sequence. Cleavage sites for each different enzyme were engineered into the GST-VP1 expression vector based on this analysis, and the fusion proteins were expressed in *E. coli* and purified by affinity chromatography. Enzymatic cleavage to release the fusion protein was performed and cleaved material was analysed using SDS-PAGE. Further capsomere purification and VLP assembly was performed, followed by qualitative and size characterisation of the VLPs. The results suggest options for improvement in the downstream processing of virus-like particle vaccines, and perhaps more broadly in the downstream processing of therapeutic proteins.

2. Materials and methods

2.1. *Bioinformatics analyses*

Python programming (Rossum and Boer, 1991) scripts were created to search through the VP1 sequence (Genbank Accession: M34958), for motifs cleaved by thrombin experimentally, as well as motifs that follow the cleavage rules outlined in Expasy Peptide Cutter program (Gasteiger et al., 2003), shown in Table 1. Positions within the motif are designated as P4, P3, P2, P1, P1′, P2′, P3′, where cleavage occurs between sites P1 and P1′ (Schechter and Berger, 1967). The scripts also scanned

Table 1 – Theoretical and experimentally observed sequences cleaved by thrombin (Chang, 1985; Gasteiger et al., 2003; Lonsdale-Eccles et al., 1980).

Source	P4	P3	P2	P1	P1′	P2′	P3′
GE Healthcare	L	V	P	R	G	S	–
Chang et al.	S	R	L	R	D	S	A
Chang et al.	S	L	S	R	L	R	–
Chang et al.	N	Y	T	R	L	R	K
Chang et al.	T	R	L	R	K	Q	M
Chang et al.	P	S	G	R	V	S	M
Chang et al.	S	M	I	K	N	L	Q
Chang et al.	P	K	L	K	W	–	–
Chang et al.	M	A	P	R	E	R	K
Chang et al.	F	F	W	K	T	F	T
Chang et al.	M	Y	P	R	G	N	H
Chang et al.	T	Y	P	R	T	N	T
Lonsdale-Eccles et al.	V	Y	A	R	V	T	A
Expasy peptide cutter	–	–	G	R	G	–	–
Expasy peptide cutter	A, F, G, I, L, T, V or M	A, F, G, I, L, T, V, W or A	P	R	not D or E	not D or E	–