Purification Process Development of Protein Subunit based Vaccine Candidates Produced using Recombinant *E. coli* Expression System- Case-Study

Part II

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Introduction to Case study

Problem: Appearance of two peaks in the purified protein product (protein Y) in RP_HPLC assay (Figure 6) and ratio changes over time.
Objective: To obtain product consistency-Protein Y

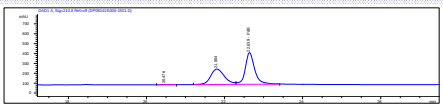
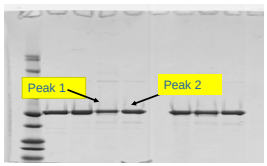


Figure 6: Purified Protein Y runs as two peaks in RP_HPLC, Peak 1 (21.8 min) has lower retention time than peak 2 (22.6 min) in RP HPLC

Challenges with two peaks in RP_HPLC

- Different batches have different peak 1/peak 2 ratio
- This ratio changes over time
- Peak 2 gets converted to peak 1 over time
- It is hard to use RP_HPLC assay to quantify and measure purity Stability indicating assay
- However, it has no impact on biological activity
- Mixture of two proteins has no impact on immunogenicity



Peak 1 appears only after during last diafiltration step in the process

SM1= 2 column purified
SM2= 3 column purified

Figure 7: SDS-PAGE analysis of Peak 1 and Peak 2 fraction showed that they have similar molecular weight

Effect of Oxidizing agents and antioxidants on the two peaks

No Treatment, 65% Peak 1, 34% Peak 2
20 mM Methionine, 4% peak 1, 95% peak 2
12 mM Thiosulfate, 13% Peak 1, 86% Peak 2

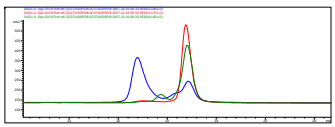


Figure 8: Diafiltration in the presence of antioxidants e.g. methionine and thiosulfate results into majority of peak 2

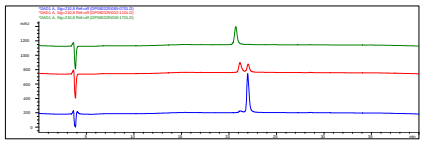


Figure 9: Protein Y can be Oxidized with Periodate treatment to Peak 1

Treatment with	%Peak1 vs. %Peak2	Biological Activity	Immunological Activity
None*	6 /94	+++	Yes
None	73 /19	+++	Yes
20 mM Methionine	4 / 96	++++	Yes
12 mM Thiosulphate	13 / 87	++++	Not done
10 mM Sodium Periodate	100 / 0	+	Yes
10 um Periodate	99 / 0	+	Yes

Figure 10: Forced Oxidized form has lower activity than reduced form, however, natural shift over time doesn't change activity

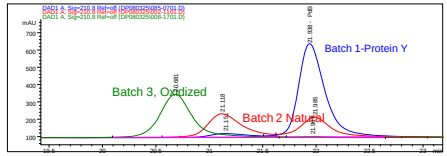


Figure 11: Oxidized Protein Y with 10 mM Periodate: shift in the retention time observed by 0.5 min on RP_HPLC

3rd column Elution	% Peak -1	% Peak 1	% Peak 2
	RT 21.4	RT 21.8	RT 22.6
No NaIO4	0	41	58
1 uM NaIO4	0	37	63
5 uM NaIO4	0	81	19
10 uM NaIO4	0	91	8
50 um NaIO4	0	100	0
250 um NaIO4	44	56	0
1 mM H2O2	0	11	89
100 mM H2O2	48	35	9

Figure 12: Titration of Oxidation reaction with different amounts of Periodate indicate that 10-50 um of Periodate is sufficient

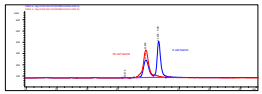


Figure 13: For 5 uM of Protein Y (1.5% is Met or 0.075 um of Met), you need 10-50 uM of NaIO4

Conclusions

- Peak 1 is more oxidized form of Peak 2
- Oxidation process is irreversible
- Peak 2 only can be generated by including oxidation inhibitors
- Peak 1 only can be generated by oxidizing agents
- Purified product made could be prepared consistently with a single peak (peak 1 or peak 2)

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