Expression of wild type and variants of human apolipoprotein A-I in Pichia pastoris

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Expression de type sauvage et des variantes de l'Apolipoprotéine A-I humaine chez *Pichia pastoris*

Expression of wild type and variants of human apolipoprotein A-I in *Pichia pastoris*

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Avant-propos / Acknowledgement

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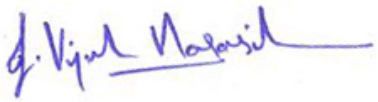

Vignesh Janakiraman

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Les lipoprotéines de haute densité (High Density Lipoprotein, HDL) permet de réduction de risque de maladies cardio-vasculaires principalement en raison de leur capacité à éliminer le cholestérol accumulé des artères (via transport inverse du cholestérol). Les effets protecteurs des HDL sont médiés par l'apolipoprotéine AI (ApoA1), qui est le La protéine la plus importante quantitativement du HDL. L'ApoA1 favorise l'efflux de cholestérol vers le foie pour l'excrétion. Une augmentation des niveaux plasmatiques de l'ApoA1 est généralement acceptée d'être cardioprotecteur, ce qui en fait un potentiel thérapeutique. Deux variantes naturelle (mutants) de l'ApoA1, Milano et Paris, sont caractérisées par une mutation ponctuelle unique a permis l'introduction d'un résidu cystéine. Populations avec ApoA1-Milano ont été rapportés d'avoir un système cardiovasculaire, même avec de faibles niveaux de plasma de ApoA1 et HDL. Il est donc d'intérêt pour générer recombinante de type sauvage et des variantes de ApoA1 humaine pour des applications thérapeutiques potentielles. Dans cette étude, de type sauvage rhApoA1 a été produit chez *P. pastoris* et purifié par chromatographie en mode mixte en une seule étape. Par la suite, un processus intégré a été le développement de la production et la récupération rapide de type sauvage rhApoA1 chez *P. pastoris* par chromatographie par lit expansée. En outre, les variantes de l'ApoA1, Milano & Paris, ont été générées par mutagenèse dirigée et ont été exprimés chez *P. pastoris*. Les motifs d'adsorption de rhApoA1-Milano et rhApoA1-Paris ont été comparés à celle de type sauvage ApoA1 et les différences ont été discutées.

Mots clés : apolipoprotéine a-I (ApoA1), *Pichia pastoris*, ApoA1-Milano et ApoA1-Paris, chromatographie par mode-mixte, HEA HyperCel, PPA HyperCel, Capto MMC

Unités de recherche

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Abstract

Expression of Wild Type and Variants of Human Apolipoprotein A-I in *Pichia pastoris*

The high-density lipoprotein (HDL) complex helps reduce the risk of cardiovascular disorders mainly due to its ability to remove accumulated cholesterol from arteries via reverse cholesterol transport. These protective effects of HDL are known to be mediated by Apolipoprotein A-I (ApoA1), which is the major protein component of HDL. ApoA1 is a lipid binding protein and promotes cholesterol efflux from peripheral tissues to the liver for excretion. An increase in the plasma levels of ApoA1 is generally accepted to be cardioprotective, making it a potential therapeutic. Two naturally occuring variants of ApoA1, namely the Milano & Paris mutants, are characterised by a single point mutation resulting in the introduction of a Cysteine residue. Populations with ApoA1-Milano have been reported to have a healthier cardiovascular system even with low plasma levels of ApoA1/HDL. It is hence of interest to generate recombinant wild type and variants of human ApoA1 for potential therapeutic applications. In this study, wild type rhApoA1 was produced in *P. pastoris* and purified by mixed-mode chromatgraphy in a single step. Subsequently, an integrated process has been development for the production and rapid recovery of wild type rhApoA1 in Pichia pastoris. This has paved way to the establishment of a scalable integrated process that could be further developed to industrial levels. In addition, the cysteine variants of ApoA1, Milano & Paris, have been generated by site directed mutagenesis and have been successfully expressed in *P. pastoris*. The binding patterns of rhApoA1-Milano and rhApoA1-Paris have been compared with that of wild-type ApoA1 and the differences have been discussed.

Keywords: apolipoprotein a-I (ApoA1), *Pichia pastoris*, ApoA1-Milano and ApoA1-Paris, mixed-mode chromatorgraphy, HEA HyperCel, PPA HyperCel, Capto MMC

Research Units

EA-4135, Biotechnologie des protéines recombinantes à visée santé, Université de Bordeaux, France

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Résumé de thèse en français

L'expression de type sauvage et des variantes de l'Apolipoprotéine A-I humaine chez *Pichia pastoris*

R.1. INTRODUCTION

R.1.1. L'INTRODUCTION GÉNÉRALE

Les troubles métaboliques, notamment le diabète, l'athérosclérose et les maladies cardiovasculaires sont les principales causes de morbidité et de mortalité dans les pays développés et en développement. L'obésité et la dyslipidémie sont les principaux facteurs de risque particulier dans les maladies cardiovasculaires (Miller, 1978).

Il est généralement accepté que les lipoprotéines de haute densité (High Density Lipoprotein, HDL) permet de réduire le risque de maladies cardio-vasculaires, car il se déplace le cholestérol au foie par le transport inverse du cholestérol (Reverse Cholesterol Transport, RCT) (Tall, Costet, & Wang, 2002). En plus, le HDL contribue également au bien-être général du système cardio-vasculaire grâce à ses propriétés anti-inflammatoires, anti-oxydantes et anti-thrombotiques (Assmann & Gotto, 2004). On pense que ces effets protecteurs des HDL être médiée principalement par l'apolipoprotéine AI (ApoA1) qui est le composant protéique majeur des HDL (Heinecke, 2010).

R.1.2. TRANSPORT INVERSE DU CHOLESTEROL

L'ApoA1 favorise l'efflux de cholestérol des tissus vers le foie (Oram, 2003) (Fig. R.1). ApoA1 prend cholestérol des cellules à travers l'ABCA1 (ATP-Binding Cassette A1) et forme des particules de HDL naissantes. Les particules HDL plus matures lors de l'activation de la Lécithine-cholestérol acyltransférase (LCAT) qui convertir le cholestérol à des esters de cholestérol (Vanloo et al., 1992). Ces esters de cholestérol de HDL sont échangés avec des triglycérides provenant d'autres lipoprotéines (LDL/VLDL) par l'action de la protéine de transfert des esters de cholestérol (Cholesteryl Ester Transfer Protein, CETP). Le transport inverse du cholestérol est complétée par le dépôt d'esters de cholestérol dans le foie, soit directement par Scavenging récepteur B1 (SR-B1) ou par LDL et le LDL-récepteur (Lewis & Rader, 2005).

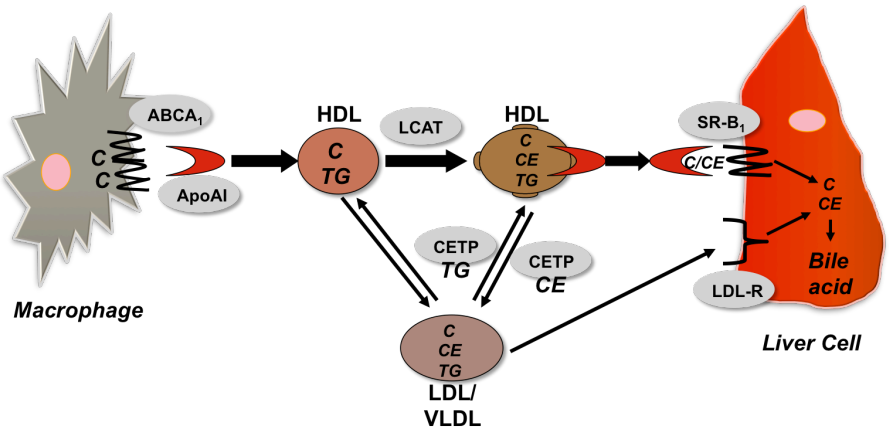


Figure R.1. Le mécanisme de transport inverse de cholestérol

On sait que des niveaux élevés dans le plasma de l'ApoA1 contribue à la réduction des risques cardio-vasculaires chez les humains (Gordon et al., 1989), ce qui rend l'ApoA1 du potentiel thérapeutique. En outre, deux variants naturels de ApoA1 : ApoA1-Milano et ApoA1-Paris ont été signalés dans les populations qui ont réduit risque d'athérosclérose (Alexander et al., 2009). Les variantes Milano et de Paris sont caractérisées par des mutations ponctuelles au R173C et R151C respectivement. L'ApoA1 de type sauvage ne contient pas de résidus de cystéine, et donc l'introduction de ces mutants cystéine dans leur permet de former des homo-dimères à liaison disulfure sur le HDL (Klon, Jones, Segrest, & Harvey, 2000).

R.1.3. LES OBJECTIVES

Dans ce travail, nous avons mis en place un processus simplifié pour générer l'ApoA1 recombinante chez *Pichia pastoris* et de la purifier par chromatographie en mode mixte.

Le système d'expression choisi pour cette étude est la levure méthylotrophe *Pichia pastoris*, en raison d'un certain nombre d'avantages qu'elle pose : la capacité d'atteindre des niveaux élevés d'expression de protéines hétérologues (Cereghino & Cregg, 2000; Sreekrishna et al., 1997), la capacité à sécréter des protéines hétérologues dans le milieu (Brake et al., 1984), et étant «généralement considéré comme sûr" (Generally Regarded As Safe, GRAS) micro-organismes (Klein, 1998). La surexpression de protéines hétérologues

chez *P. pastoris* est atteinte par le promoteur AOX1 qui est activé dans des conditions de carbone faim (Daly & Hearn, 2005).

Après l'expression, l'ApoA1 recombinante a été ensuite purifié par Chromatographie en mode mixte. Chromatographie par mode mixte ou « multimodal » sont généralement émis l'hypothèse d'agir par une combinaison d'interactions électrostatiques et hydrophobes (Chung, Freed, Holstein, McCallum, & Cramer, 2010).

Donc, les objectifs suivants sont envisagés dans ce travail de thèse:

- Clonage et expresion de type sauvage l'ApoA1 recombinante (rhApoA1) dans *P. pastoris* X-33
- Purification de rhApoA1 de la culture de *P. pastoris*, et scale-up de la purification à grande échelle avec l'adsorption a lit expansée (Expanded Bed Adsorption, EBA)
- Le clonage, l'expression et la purification de rhApoA1 chez *P. pastoris* SMD-1168 (souche de *P. pastoris* déficiente en protéase)
- Génération de variantes de l'ApoA1: Milano (R173C) et Paris (R151C)

R.2. CLONAGE ET EXPRESSION DE TYPE SAUVAGE DE L'ApoA1 HUMAINE RECOMBINANTE DANS *P. pastoris* X-33

R.2.1. MÉTHODES

La séquence correspondant à l'ApoA1 a été amplifié en utilisant des amorces spécifiques sa séquence, et a été clone dans le vecteur pPICZαA et transformé en *Pichia pastoris* X-33 compétente, en suite les transformants ont été sélectionnés la résistance à la Zéocine™ (jusqu'à 2 mg / ml).

Un étude préliminaire d'expression a été réalisée sur plusieurs clones de haute résistance sur les cultures en flasques, avec induction effectuée en utilisant 0,5% de méthanol chaque 24h pour 120hrs. Par la suite, le clone exprimant le plus élevé a été en outre pour l'expression dans un bioréacteur de 2L dans conditions régulées: Temp 30°C, pH 6,0, 15% saturation d'oxygène, induction avec 0,5% de

méthanol chaque 12hrs. L'expression a été analysée par SDS-PAGE, dot-blot et Western blot.

R.2.2. RÉSULTATS ET DISCUSSION

Après le clonage, le construit (pPICZα-ApoA1) a été soumis à un séquençage d'ADN et a été vérifiée. La construit ete transforme par électroporation dans compétentes cellules de Pichia pastoris X-33, un certain nombre de transformants présentait une haute résistance à la zéocine et cinq d'entre eux ont été repris pour les études d'expression sur les cultures en flacons.

Suite les études sur les cultures en flacons, un clone a été prise pour l'expression dans un bioréacteur. Une colonie unique a été inoculée et cultivée dans des flacons à déflecteurs de 100mL de milieu de glycérol-complexe (Buffered Complex Glycerol Medium, BMGY) jusqu'à ce que la DO (600 nm) atteint de 4 au 8, ce qui a été inoculé dans 2 litres d'BMGY dans un BIOSTAT® Bplus 2l bioréacteur. Les paramètres ont été maintenus constamment, et lors de la consommation complète du glycérol dans le milieu, une phase méthanol fed-batch a été initiée par l'addition de methanol toutes les 12 heures jusqu'à une concentration finale de 0,5% pour 120hrs. L'expression des protéines a été vérifiée par dot-blot, SDS-PAGE et analyse western blot.

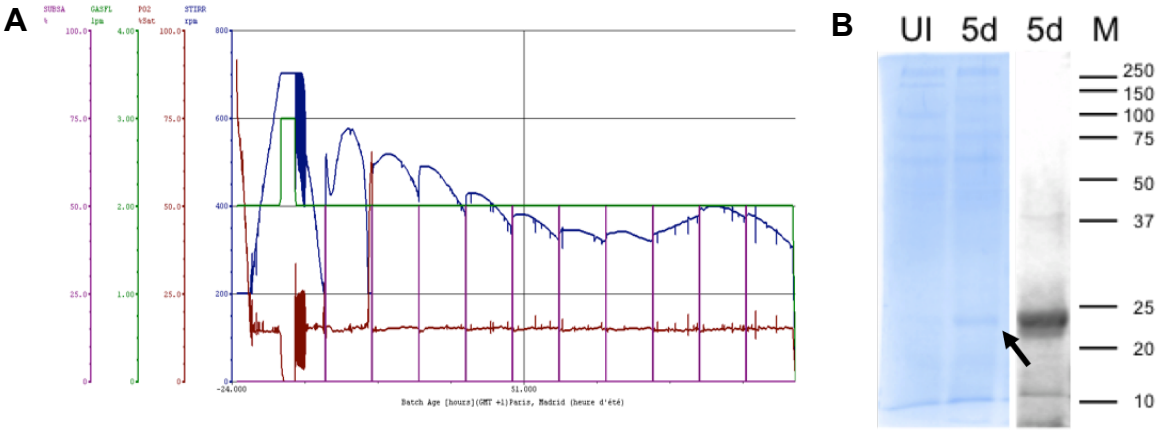


Figure R.2. Profil (A) et l'analyse SDS-PAGE et western blot (B) de l'expression de rhApoA1 expression in *P. pastoris* X-33.

Après vérification de l'expression, la milieu de *P. pastoris* contenant rhApoA1 a été utilisé pour les expériences de purification.

R.3. PURIFICATION DE TYPE SAUVAGE rhApoA1 EXPRIMÉE CHEZ *P. pastoris* X-33 PAR CHROMATOGRAPHIE EN MODE MIXTE

R.3.1. MÉTHODES

Un certain nombre de méthodes de purification publiés ont été testés pour leur aptitude à récupérer rhApoA1 exprimée dans *P. pastoris* (Feng, Cai, Song, Dong, & Zhou, 2006; Marco Aurélio Zezzi Arruda, Lopes, Marcelo Anselmo Oseas da Silva, & Gozzo, 2011). Ces méthodes ont été conservés en tant que méthodes de référence pour évaluer l'efficacité des procédés de purification développé dans cette étude.

Deux méthodes de chromatographie en mode mixte ont été testés pour leur capacité à capturer rhApoA1 directement à partir du milieu d'expression de *P. pastoris* : HEA HyperCel (Pall Life Sciences) et Capto MMC (GE Healthcare), dont les structures sont à la Fig. R.3. Les conditions de chromatographiques ont été établies sur la base des directives de leur fabricant respectif, et les différentes fractions recueillies ont été analysées en utilisant SDS-PAGE et techniques Western blot.

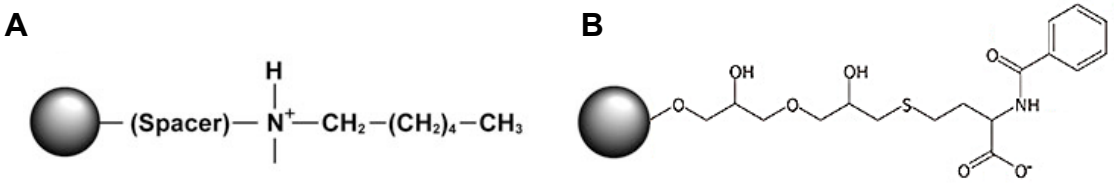


Figure R.3. Structures de ligands (A) HEA HyperCel et (B) Capto™ MMC

Après avoir optimisé avec succès conditions chromatographiques pour récupérer rhApoA1, les protéines purifiées ont été digérés par la trypsine et analysées sur un-ESI-Q-TOF LC spectromètre de masse (modèle Agilent G6540A) pour évaluer la couverture de la séquence, afin de vérifier la protéine purifiée .

R.3.2. RÉSULTATS ET DISCUSSION

Des méthodes publiées pour la purification de l'ApoA1 ont été adaptées pour travailler avec de milieu de *P. pastoris*. Après les trois procédés décrits ont été testés, leur efficacité pour « scale-up » a été évaluée. Étant donné que toutes les méthodes précédemment rapportées ne sont pas réalisables pour scale-up, il était impératif d'explorer d'autres procédés chromatographiques de colonnes

efficaces qui pourraient être utilisés pour purifier l'rhApoA1 exprimée chez *P. pastoris.*.

R.3.2.1. PURIFICATION DE L'ApoA1 AVEC HEA HYPERCEL

La résine en mode mixte HEA HyperCel généralement activé l'adsorption et l'élution de protéines par attraction hydrophobe et répulsion électrostatique, respectivement. En cas de rhApoA1, l'adsorption a été réalisé à pH neutre avec moins conductivité promouvoir l'attraction hydrophobe. Par la suite, le sel a été éliminé et le pH a été réduit, ce qui pourrait avoir exposé charges positives sur la surface de la protéine qui faciliter élution par répulsion électrostatique. Le profil de purification est rapporté dans Fig. R.4.

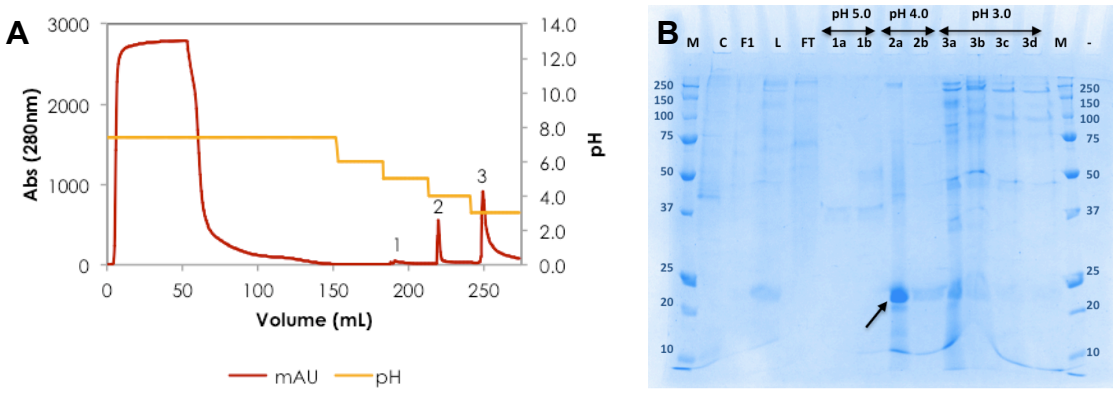


Figure R.4. Purification de l'rhApoA1 a l'aide de l'HEA HyperCel : (A) Chromatogram et (B) analyse de SDS-PAGE 12%.

Un désavantage de cette méthode était l'emploi de pH bas (4,0) pour éluée de l'ApoA1, comme il est connu que ApoA1 forme amyloïdes aux basses pH. (Ramella et al. 2012).

R.3.2.2. PURIFICATION DE L'ApoA1 AVEC CAPTO™ MMC

Le ligand Capto™ MMC favorise l'adsorption des protéines par des interactions hydrophobes, ioniques et thiophiles. Comme il marche comme un échangeur de cations faible, il a été prévu pour fonctionner d'une manière inverse à la HEA HyperCel (qui contient un groupe à charge positive). Comme l'adsorption a été effectuée à pH 5,0 (le pH du milieu d'expression de *P. pastoris*), aucun prétraitement de l'échantillon était nécessaire. En outre, la protéine liée a été

élue à pH neutre (Fig. R.5), de faciliter le maintien de la fonctionnalité maximale. Ce processus était plus compatible pour scale-up et pour la développement d'un procède industrielle.

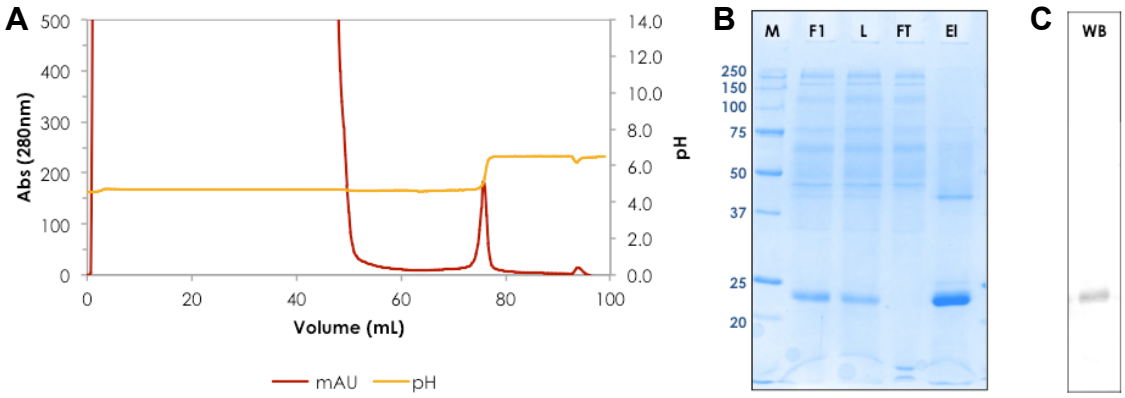


Figure R.5. Purification de l'rhApoA1 a l'aide de Capto MMC : (A) Chromatogram, (B) analyse de SDS-PAGE 12% et (C) Blot western

R.3.2.3. COMPARAISON DES MÉTHODES DE PURIFICATION AVEC MÉTHODES PUBLIÉES

Les méthodes chromatographiques développés dans ce travail ont été comparées avec les méthodes déjà publiées pour la purification de rhApoA1 (Tableau R.1).

Tableau R.1. Comparaison des méthodes de purification

	Méthode de purification	Nombres d'étapes	Rendement	Purité d'rhApoA1	Reference
1.	Extraction par « Cloud-point » avec Triton X-114	2	55.97%	57.1%	(Marco Aurélio Zezzi Arruda et al., 2011)
2.	Précipitation avec acétone froid	14	60.00%	71.9%	(Feng et al., 2006)
3.	Chromatographie par mode mixte (HEA HyperCel)	1	56.25%	70.2%	*Présente travail*
4.	Chromatographie par mode mixte (PPA HyperCel)	1	52.50%	76.3%	*Présente travail*
5.	Chromatographie par mode mixte (CaptoMMC)	1	68.89%	84.0%	*Présente travail*

Il était évident que les deux méthodes de purification développées dans cette étude ont été mieux par rapport aux méthodes déjà publiées.

R.3.2.4. IDENTIFICATION DE L'ApoA1 PURIFIÉE PAR SPECTROMÉTRIE DE MASSE

La rhApoA1 purifié par les deux procédés de purification ont été digérés avec trypsine et analysée par spectrométrie de mass sur ESI-Q-TOF MS/MS (Agilent). La rhApoA1 purifié par des deux procédés montré couverture de séquence substantielle (~ 65%) avec la séquence d'ApoA1 humaine disponible sur NCBI. Ce fut une validation globale des méthodes de purification utilisées. Bien que la protéine purifiée a été vérifié pour être ApoA1, le poids moléculaire sur des gels SDS-PAGE a été constamment inférieur (~ 25-26kDa) que le poids moléculaire attendue (28 kDa).

Cela a conduit à la spéculation sur une éventuelle troncature de la protéine due à l'activité de la protéase de *P. pastoris*. Pour vérifier cette hypothese, le gène de l'ApoA1 a ensuite été transformé en une souche déficiente de la protéase de *P. pastoris* (SMD1168) et a induit avec du méthanol pour exprimer rhApoA1. Les détails expérimentaux et les résultats sont discutés en détail dans la section 4.

R.4. CLONAGE, L'EXPRESSION ET LA PURIFICATION DE rhApoA1 CHEZ *P. pastoris* SOUCHE SMD-1168 (DEFICIENTE EN PROTEASE)

Le poids moléculaire de rhApoA1 obtenu lors de l'expression de type sauvage souche *P. pastoris* X-33 a été constamment inférieur au poids moléculaire attendu, au cours des analyses SDS-PAGE. Nous regardé si il y avait une activité de protéase conduisant à cette troncature hypothétique, et essayé à exprimer la rhApoA1 dans une souche déficiente en protéase de *P. pastoris* : SMD1168. Les protéines exprimées étaient comparées entre les deux souches, de conclure globalement si la protéine a en effet été tronquée.

R.4.1. MÉTHODES

Compétente *P. pastoris* SMD1168 cellules ont été préparés et la construit pPICZα-ApoA1 a été transformé. Les transformants présentant une forte

résistance à la Zéocine ont été choisis pour analyse de l'expression. L'expression a été effectuée d'une manière similaire à celle de la souche de type sauvage (X-33). Les niveaux d'expression ont été analysés par SDS-PAGE.

Suite de la production, l'rhApoA1 exprimé de *P. pastoris* SMD1168 a été purifié par chromatographie en mode mixte avec de ligand Capto™ MMC. Le procédé de purification était similaire que la procédé developpe pour la rhApoA1 exprimé sur le *P. pastoris* type sauvage, et les protéines purifiées à partir de APOA1 deux souches ont été comparées pour leur taille et leur séquence.

R.4.2. RÉSULTATS ET DISCUSSION

L'expression de rhApoA1 chez *P. pastoris* SMD1168 a été vérifiée par SDS-PAGE. Aucune différence significative du poids moléculaire été observée entre la rhApoA1 exprimée par X-33 et SMD-1168.

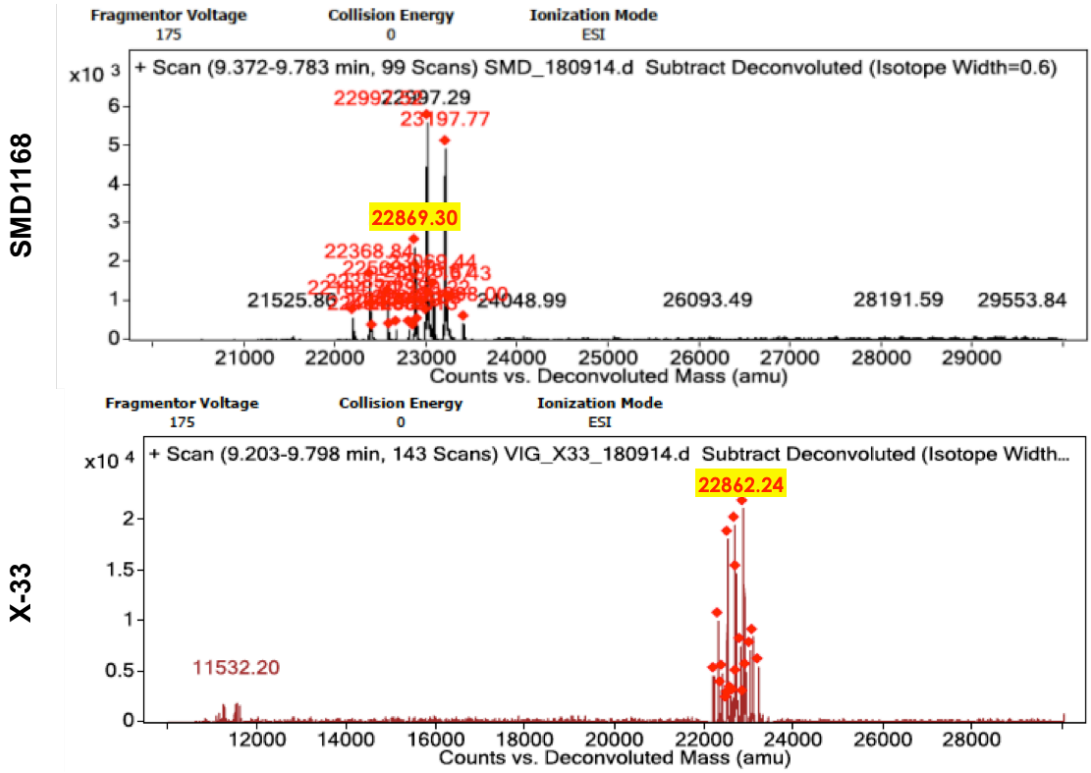


Figure R.6. Analyse spectrométrie en mass (ESI-Q-TOF) de fractions contenant la rhApoA1 purifiées à partir *P. pastoris* X-33 et SMD1168

La profil de purification de la rhApoA1 chez *P. pastoris* SMD1168 à l'aide Capto™ MMC a été similaires de purification profil obtenu en purifiant rhApoA1 de *P. pastoris* X-33.

En analyse par spectrométrie en mass, les rhApoA1 purifiée à partir de deux souches des *P. pastoris*, il a été observé qu'il n'y avait pas de différence significative entre les poids moléculaires des deux souches de rhApoA1 (Fig. R.6). Cela a conduit à croire que la protéine était en fait complète et intact.

R.5. MISE A L'ECHELLE DE PRODUCTION ET DE PURIFICATION DE rhApoA1 CHEZ *P. pastoris* X-33

R.5.1. MÉTHODES

Suite a l'expression et la purification de rhApoA1 en échelle laboratoire, les études pour la mise à l'échelle de la production et la purification de rhApoA1 a été envisagé. La première étape vers l'intensification de l'expression a été effectuée dans un bioréacteur de 5l capacité. Les paramètres de l'expression ont été réglés comme ils l'étaient dans les lots de bioréacteurs 2l (voir section 2.1), et l'expression des protéines ont été analysées par SDS-PAGE et dot-blot.

À la fin du lot de production, toute la culture de *P. pastoris* a été passé à travers une colonne équilibrée directe CST-I en mode "adsorption par lit expansé". Les conditions de tampon ont été maintenues comme il été optimisée dans des colonnes a l'echelle laboratoire, et le profil a été surveillée par l'absorbance à 280 nm. Les différentes fractions ont été recueillies et analysées par SDS-PAGE.

R.5.2. RÉSULTATS ET DISCUSSION

La profil de production de la rhApoA1 a ete suivre par SDS-PAGE et les niveaux de production de la rhApoA1 été comparables ont obtenus dans le bioréacteur de 2l (section R.2.2). Un taux d'addition de méthanol plus fréquente (chaque 8 heures) assurée l'induction suffisant de cellules en croissance. L'examen microscopique périodiques d'échantillons a vérifié l'absence de contamination dans la culture. La production en bioréacteur était très reproductible.

Tableau R.2. Comparaison des échelles de l'expression de la rhApoA1

Paramètre\Échelle	Flasque	Bioréacteur 2L	Bioréacteur 5L
Volume de culture	150 mL	1800 mL	4000 mL
DO_{600nm} finale	27.56	41.34	44.71
pH d'induction	6,0	5,0	5,0
Système de tampon	Phosphate	phosphate + acide/base	phosphate + acide/base
Température de l'induction	30°C	28°C	28°C
Rendement de la rhApoA1	22,4 mg/l	37,5 mg/l	43,8 mg/l

Le profil chromatographique de la purification (Fig. R.7) en utilisant Direct CST-I (la même chimie de ligand comme Capto™ MMC), était similaire à celle obtenue dans des conditions à colonne paquée (Section R.3.2). Analyse par SDS-PAGE a confirmé la capture de tous rhApoA1 du milieu à une très haute concentration.

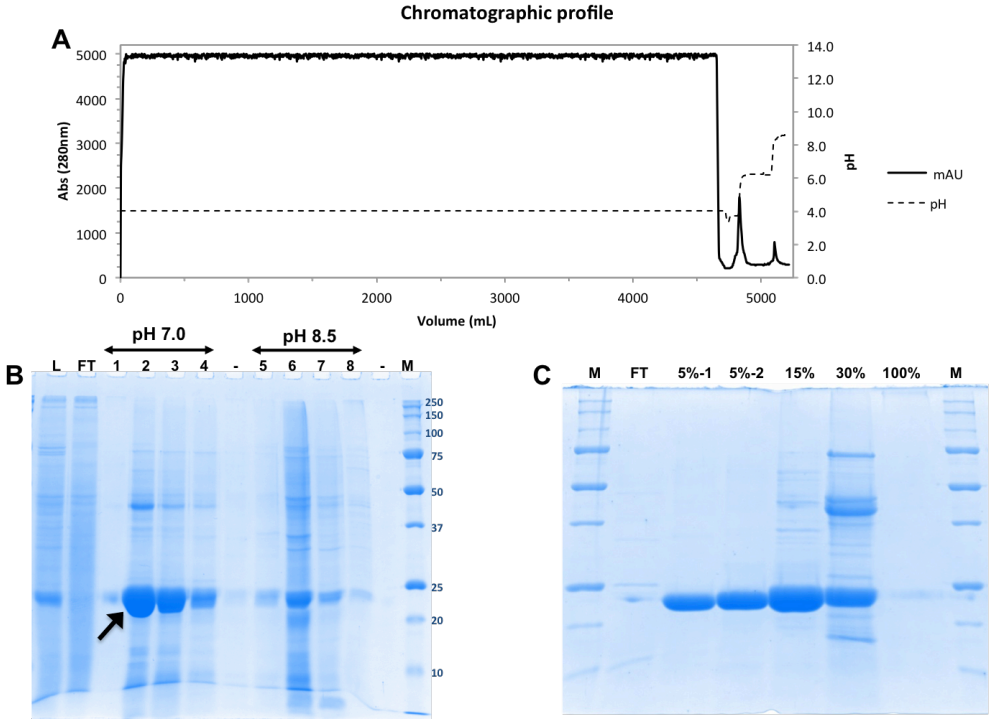


Figure R.7. Purification de rhApoA1 par Direct CST-I en mode « adsorption lit expansee ». (A) Chromatogramme et (B) 12% SDS-PAGE de la charge (L), les fractions non retenues (FT) et éluée à pH 7,0 (fractions 1 - 4) et 8,5 (fractions 5 - 8). (C) 12% de l'analyse SDS-PAGE de fractions provenant Resource Q échangeuse d'ions de polissage étape: charge (L),

non retenu (FT), et les fractions éluées à 5%, 15%, 30% et 100% de tampon d'élution (20 mM de tampon phosphate, pH 7,0, 1M NaCl).

Quelques impuretés ont été éliminées par une deuxième étape de chromatographie avec échange d'anions. Ce processus de purification évolutive montre très prometteur pour la production industrielle à grande échelle de rhApoA1.

R.6. GENERATION DE VARIANTES DE L'ApoA1 : MILANO & PARIS

Deux variantes naturelles de l'ApoA1, Milano et Paris, sont caractérisés par une seule substitution de la pointe de l'arginine à cystéine à positions différentes. Ces deux variantes ont été rapportées chez des populations avec un risque réduit de troubles cardio-vasculaires. Une étude comparative de ces variantes pourrait donner une meilleure idée de leur mécanisme d'action, et aider à générer potentiellement thérapeutiques.

R.6.1. MÉTHODES

La mutagenèse dirigée a été utilisée pour introduire des mutations dans le gène de la rhApoA1. Les Milano (R173C) et Paris (R151C) variantes ont été générés en utilisant des amorces spécifiques de la séquence d'intégrer les mutations souhaitées. Les constructions ont été ensuite transformé par électroporation dans des cellules compétentes de *P. pastoris* X-33, et les transformants ont été criblés pour la résistance à la Zéocine. Quelques clones ont ensuite été essayé pour l'expression par induction avec du méthanol en cultures flacons et le bioréacteur 2L, et l'expression a été suivre par dot-blot.

Suite de l'expression, des expériences préliminaires de purification ont été réalisées pour évaluer les différences dans l'adsorption des variantes de l'ApoA1 au ligand Capto™ MMC en mode mixte.

R.6.2. RÉSULTATS ET DISCUSSION

Les constructions de l'ApoA1-Milano et l'ApoA1-Paris ont été générées par mutagénèse dirigée, et vérifiés par séquençage d'ADN. Par la suite, les

constructions ont été transformés de individuellement dans compétentes X-33 cellules de *P. pastoris* et les transformants en résistante à 2 mg/mL Zéocine ont été sélectionnés pour les études de l'expression. Trois clones chacun de Milan et de Paris ont été testés dans des cultures en ballon agité, et tous ont montré expression réussie de variantes rhApoA1 (Fig. R.8). Un clone de chaque ont ensuite été poussée plus loin pour l'expression au niveau de bioréacteur 2L.

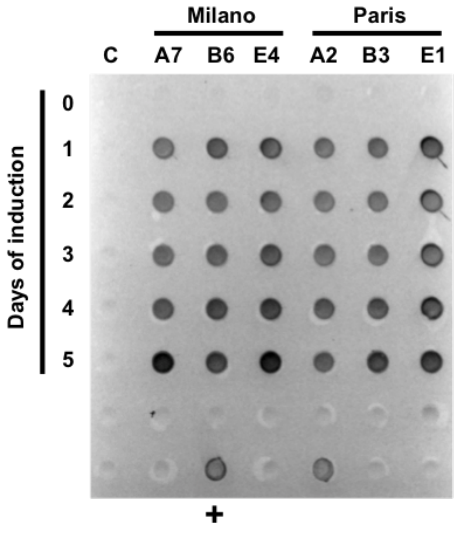


Figure R.8. Analyse « Dot-Blot » de l'expression de l'ApoA1-Milano et l'ApoA1-Paris surexprimé chez *P. pastoris* X-33

Purification préliminaire des deux variantes de l'ApoA1, Milano et Paris ont été testés à l'aide de deux supports de chromatographie HEA HyperCel et Capto MMC. Les profils d'adsorption de l'ApoA1-Milano et l'ApoA1-Paris étaient significativement différents de l'ApoA1 type sauvage. Ce permis de mieux comprendre les changements importants dans la structure de liaison induite-cystéine à partir du résidu cystéine introduit.

R.7. CONCLUSION

Cette thèse a focalisé sur la génération de type sauvage et des variantes de l'apolipoprotéine AI humaine chez la levure *Pichia pastoris*. La méthode de production et de purification de type sauvage de l'ApoA1 était mise en échelle. La purification de l'ApoA1 été fait par l'adsorption en lit expansée (*Expanded Bed Adsorption*, EBA). La comparaison des ApoA1 exprimé en type sauvage *P. pastoris* X-33 et la souche protéase déficiente *P. pastoris* SMD1168 ont confirmé la production de rhApoA1 complet. Les études sur la génération de variantes

Milano et Paris de ApoA1 ouvre de nouvelles avenues pour effectuer des études comparatives entre le type sauvage et des mutantes de l'ApoA1.

Chapter 1

General Introduction & Review of Literature

1.1. GENERAL INTRODUCTION

1.1.1. CARDIOVASCULAR DISORDERS AND ATHEROSCLEROSIS

With great advances in healthcare, the major causes of death in the industrialised world have shifted from infectious diseases to degenerative ones such as cardiovascular disorders (CVD), this shift being termed as "the epidemiologic transition" (Yusuf et al. 2001). Over the years, studies have reflected on the migration of the global burden of Ischemic Heart Diseases from high-income countries to middle- & low-income countries, India included (Finegold, Asaria, and Francis 2013). Indians are one of the more vulnerable populations in the world, and deaths due to CVD/stroke is very high due to a number of factors, not limiting to genetic factors, lifestyle habits and inadequate healthcare policies (Reddy and Yusuf 1998). Owing to the rising number of CVD cases, India is slated to become the world's CVD capital within the next ten years (Gupta et al. 2008).

Atherosclerosis is one of the major components of CVD, and is characterised by the hardening of arteries due to invasion and accumulation of macrophages and subsequent build-up of cholesterol, lipids and lipoproteins (Epstein and Ross 1999). Advanced stages of atherosclerosis often occlude blood flow causing several mortal conditions such as myocardial infarction, cardiovascular stroke, or form emboli that could affect other tissues. One of the major causative reasons for the progression of atheroma is the dysregulation of cholesterol metabolism in the body (Assmann and Gotto 2004).

1.1.2. CHOLESTEROL AND ITS METABOLISM

Cholesterol is a sterol molecule, which is an essential structural component of higher eukaryotic cells, in addition to being a precursor molecule for the biosynthesis of steroid hormones, bile acids, etc. (Hanukoglu 1992). Cholesterol molecules are used to modulate the membrane fluidity of cells: by varying their concentration with changes in temperature. The structure of cholesterol has been elucidated in Fig. 1.1.

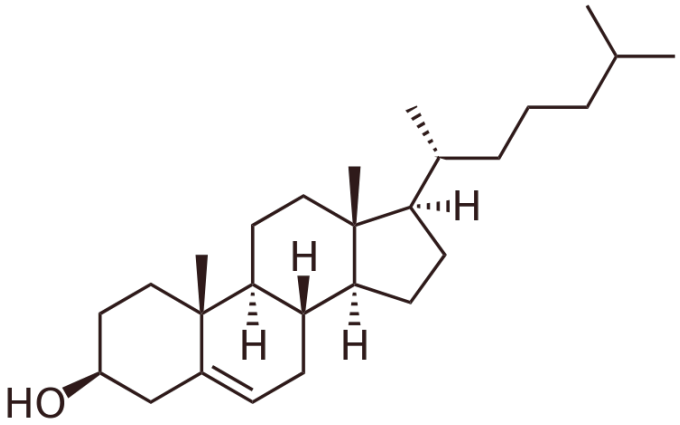


Figure 1.1. Structure of Cholesterol (Reproduced from Wikipedia EN: http://en.wikipedia.org/wiki/Cholesterol)

The cholesterol molecule contains a hydroxyl group that enables it to interact with the polar heads of membrane phospholipids and sphingolipids; whereas the bulky steroid and hydrocarbon chain sink into the membrane along with they hydrophobic fatty acid chains of lipids (Yeagle 1991). This ability of cholesterol to blend in with membrane lipids enables it to provide fluidity to the cell membrane when required. In addition to providing membrane fluidity, cholesterol is also a precursor molecule for the synthesis of steroid hormones and bile acid (Berg et al. 2002).

The average cholesterol intake is approximately 400mg per day, with primary sources being *de novo* biosynthesis and diet. About 50% of the cholesterol from dietary sources is actually absorbed (the balance is directly excreted), and hence there is a bulk production through biosynthesis pathways for catering to the body's cholesterol requirements (Lehninger, Nelson, and Cox 2005).

As cholesterol is only mildly soluble in water, it is primarily transported in blood through protein carriers called lipoproteins, which are complex structures with a hydrophilic exterior (with polar lipid heads and proteins) and an apolar core (explained in greater detail in Section 1.2). Lipoproteins are classified based on their density, and vary in protein compositions and functions.

1.2. LIPOPROTEINS

Lipoproteins are complex biological assemblies of lipids and proteins that enable the transport of fatty acids and cholesterol through water inside and outside cells (Jonas 2002). The proteins enable the emulsification of lipid particles, facilitating the smooth movement of cholesterol and triglycerides through blood.

1.2.1. STRUCTURE AND CLASSIFICATION OF LIPOPROTEINS

Lipoprotein molecules vary from discoidal to spherical forms, with a hydrophobic core and a hydrophilic surface (Jonas 2002).

As marked in Fig. 1.2, the proteins and the hydrophilic head of lipid molecules form the polar outer shell encapsulating the hydrophobic core made of cholesterol, fatty acids and triglycerides. The most important protein component of lipoproteins is apoproteins. Apoproteins bind to lipids to form nascent lipoproteins (Jackson, Morrisett, and Gotto 1976). There are a number of apoproteins associated with various sizes of lipoproteins; for example, apolipoprotein B associates with LDL and chylomicrons and apolipoprotein A associates with HDL.

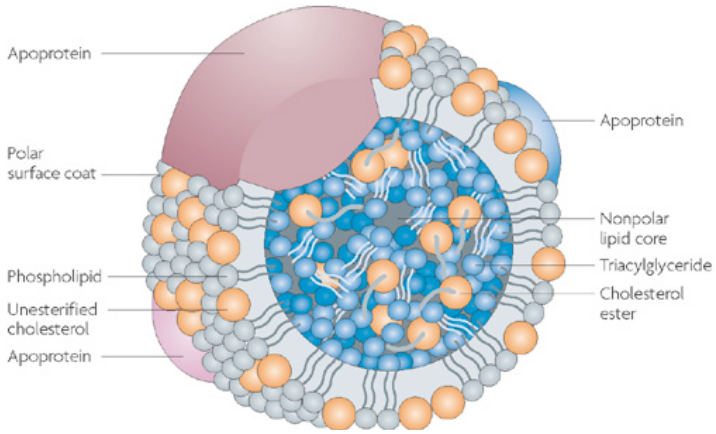


Figure 1.2. Lipoprotein structure highlighting components comprising hydrophilic surface (proteins, polar lipid head) and hydrophobic core (non-polar lipid, cholesterol, cholesterylester, triglycerides and fatty acids), reproduced from (Wasan et al. 2008).

Lipoproteins can be classified based on three different parameters:

(i) Based on density/size

(ii) Based on electrophoretic mobility

(iii) Based on nature of Apo- protein content

Among the above classification methods, classification of lipoproteins based on their density is most commonly adopted. The density of lipoproteins decreases with an increase in the ratio of lipids to proteins in them. Based on the density of the lipoprotein molecule, they are classified into high-density lipoproteins (HDL), low-density lipoproteins (LDL), intermediate-density lipoproteins (IDL), very low-density lipoproteins (VLDL) and chylomycrons. The relative sizes and densities of these various lipoproteins have been highlighted in Fig. 1.3.

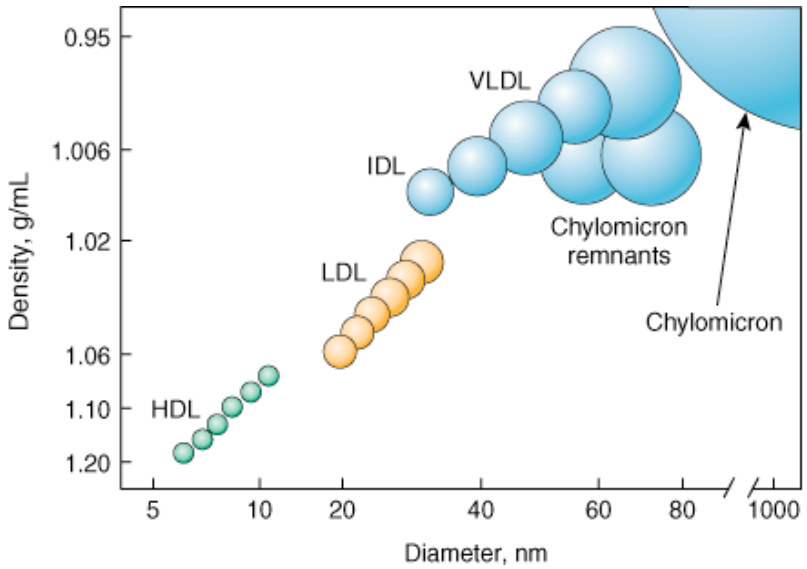


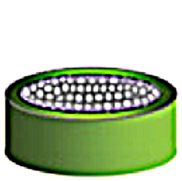
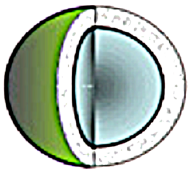
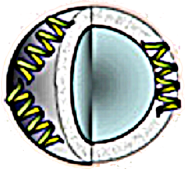
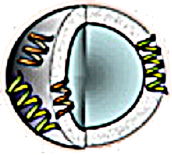
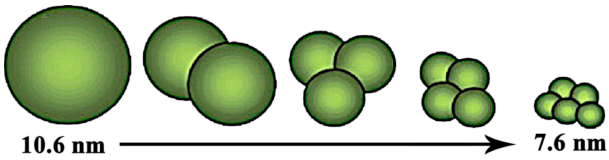
Figure 1.3. Classification of lipoproteins based on diameters (nm) and density (g/ml).

The lipoproteins of various densities play key roles in cholesterol metabolism in the body. Chylomycron are created from intestinal absorption of triacylglycerol & other lipids, VLDL/LDL particles are derived from the liver for the export of cholesterol, and HDL particles are formed from cholesterol effluxed from peripheral cells such as macrophages. There is a constant exchange of cholesterol, cholesterol-esters and triglycerides between lipoproteins, which play a key role in regulating cholesterol homeostasis.

1.2.2. HIGH-DENSITY LIPOPROTEINS (HDL)

The High Density Lipoprotein, or HDL, is the densest and smallest of lipoproteins. HDL particles range from 7 to 12nm in diameter, and from 1.063 to 1.25 g/ml in density (P. Barter et al. 2003).

Table 1.1. Classification of High Density Lipoprotein (HDL)

S. No.	Parameter for classification	Sub-populations (Tabet and Rye 2009)
1.	Shape of the HDL particle	 Discoidal HDL  Spherical HDL
2.	Composition of Apolipoproteins	 A-I HDL  A-I/A-II HDL
3.	Size of the HDL particle	
4.	Density of HDL particle	**HDL_2**: 1.063 < d < 1.125 g/ml **HDL_3**: 1.125 < d < 1.210 g/ml
5.	Electrophoretic Mobility	**Pre-β HDL**: lipid-poor/lipid-free ApoA1 & discoidal HDL **α-HDL**: Spherical HDL particles (HDL_2 & HDL_3) **γ-HDL**: Large spherical particles with ApoE

Compared to other lipoproteins, HDLs have the highest proportion of proteins (>50%) relative to their lipid content. The hydrophobic core of HDL is mainly composed of cholesterol/cholesterol-esters and a small amount of triglyceride (Rifai, Warnick, and Dominiczak 2000). The major lipid composition of HDL includes phospholipids (50%), cholesteryl esters (30%), free cholesterol (10%) and triglycerides (10%).

One of the key characteristics of HDL is their heterogeneity: in size, density and apolipoprotein composition. HDLs are classified into five sub-populations according to their size (row 3, Table 1.1), and two major subfractions based on their density (row 4, Table 1.1). Furthermore, they are also classified based on

their apolipoprotein composition into those that contain only ApoA1 (A-I HDL) and ones that contain both ApoA1 & ApoA2 (A-I/A-II HDL), highlighted in row 2 (Table 1.1).

HDL is generally perceived as cardio-protective, primarily due to its role in Reverse Cholesterol Transport (RCT), in addition to anti-oxidant, anti-inflammatory, anti-apoptotic, anti-thrombotic and anti-platelet activating effects (Tabet and Rye 2009).

1.2.3. REVERSE CHOLESTEROL TRANSPORT (RCT)

The process by which cholesterol is moved from peripheral tissues to the liver for catabolism is known as Reverse Cholesterol Transport (RCT, Fig. 1.4). RCT is the major cardioprotective function of the HDL particle (Fielding and Fielding 1995).

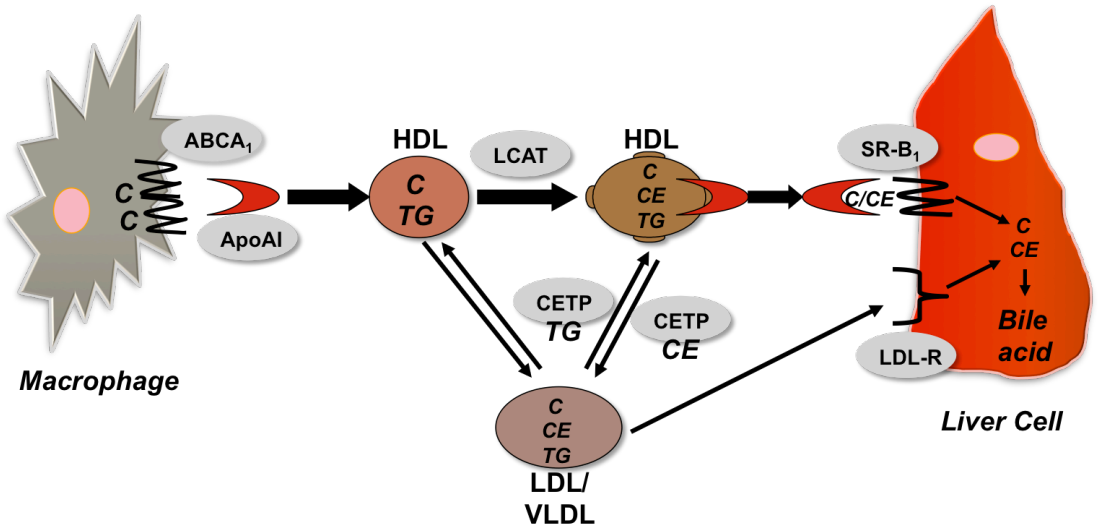


Figure 1.4. Schematic of Reverse Cholesterol Transport (RCT)

Briefly, either lipid-free ApoA1 or lipid-poor pre-β HDL particles (from liver or intestine) accept cholesterol from macrophages through the effluxor ABCA1 (ATP-Binding Cassette A1) to form discoidal HDL particles. This discoidal HDL particle accepts further cholesterol and trigliceride molecules to form a spherical HDL molecule. The key step in RCT is the activation of HDL by Lecithin-Cholesterol Acyl Transferase (LCAT), an enzyme that catalyses the conversion of cholesterol to cholesterylester, resulting in the maturation of HDL (Vanloo et al. 1992). From the mature HDL, there are two routes for the cholesterol to reach

the liver: one directly through Scavenging Receptor B1 (SR-B1); second through the LDL to LDL-receptor. The exchange of cholesterol/cholsesterylester/triglycerides is achieved by the action of Cholesteryl Ester Transfer Protein (CETP), which is a key actor in maintaining the ratio of HDL to LDL in the blood (P. J. Barter et al. 2003). Once cholesterol/ cholesterylester reaches the liver, it is subsequently converted to bile acid and excreted via feces.

The RCT represents one of the key pathways by which cholesterol homeostasis is maintained in the body, and a number of interventions have been proposed to intervene/enhance various stages of this pathway, which will be discussed in greater detail in the following sub-section.

1.2.4. LIPOPROTEIN RATIO & CARDIOVASCULAR WELLBEING

Over the decades, several epidemiological studies have demonstrated that increasing levels of HDL are inversely correlated to risk of CHD/CVD and atherosclerosis (Assmann and Gotto 2004; P. Barter et al. 2003; William B. Kannel et al. 1971). The Framingham Study was the first to report a correlation between serum cholesterol levels and coronary heart disease risk (William B. Kannel et al. 1971), classifying cardiovascular risk based on the ratio of total cholesterol to HDL-cholesterol (W. B. Kannel 1983). This has been subsequently followed by numerous epidemiological and clinical studies across the globe, resulting in the inclusion of HDL-C in the list of risk-predictors for atherosclerosis and cardiovascular disorders (Assmann and Gotto 2004). It is estimated that a 1mg/dl increase in HDL-cholesterol correlates to a 2-3% reduction in the risk of cardiovascular events (Gordon et al. 1989).

On the other hand, high levels of LDL and LDL-C (LDL cholesterol) were correlated with an increased risk of CHD/CVD. However, risk factors based on absolute values of LDL/LDL-C weren't sufficient to accurately predict disposition to CHD, especially for patients with intermediate risk (Superko and King 2008). Subsequently, ratios of total cholesterol (TC) to HDL cholesterol (HDL-C), and LDL-C to HDL-C were considered to provide a more reliable risk assessment.

Typical risk levels and target levels to be achieved to bring down risk have been summarised in Table 1.2.

Table 1.2. Risk categories and target levels for CHD (Millán et al. 2009)**.**

Ratio	Primary prevention				Secondary prevention			
	Risk level		Target		Risk level		Target	
	Men	Women	Men	Women	Men	Women	Men	Women
TC/HDL-C	>5.0	>4.5	<4.5	<4.0	>4.0	>3.5	<3.5	<3.0
LDL-C/HDL-C	>3.5	>3.0	<3.0	<2.5	>3.0	>2.5	<2.5	<2.0
ApoB/ApoA-I	>1.0	>0.9	<0.9	<0.8	>0.8	>0.7	<0.7	<0.6

A key point to note in table 1.2 is the distinction between primary and secondary prevention ratios. In persons without established CHD, risk levels and target ratios are as defined under primary prevention. Once a CHD event has taken place, there is a significant increase in cardiovascular risk, and hence the target levels for various parameters are much lower than for primary prevention persons (Grundy et al. 1999).

Although absolute values and ratios of various lipoproteins and cholesterol prove to be a reasonable indicator for cardiovascular risk, there have been several exceptions to it. Some studies have reported the occurrence of CVD in certain individuals despite high levels of HDL (Manninen et al. 1992).

Furthermore, reports at the beginning of this millennium showcased the propensity of HDL and its major protein component apolipoprotein A-I to undergo oxidation (Lemin Zheng 2004; Panzenböck et al. 2000; Shao et al. 2008). Oxidised ApoA1/HDL showed reduced ability in cholesterol binding and LCAT activation. In addition, for several decades, studies have shown the presence of better quality ApoA1/HDL in certain population, through naturally occurring mutations (Alexander et al. 2009; Weisgraber et al. 1983). This brought in a paradigm shift in thinking that the mere quantity of HDL wasn't sufficient for determining cardiovascular risk, but the quality of the HDL particle also had to be considered for determining risk. Subsequently, a number of diagnostic targets have been envisaged, which have been detailed in the next section (Smith 2010).

1.3. APOLIPOPROTEIN A-I

Human Apolipoprotein A-I (ApoA1) is the major protein component of the HDL, constituting about 70% of the total HDL protein (Rogers et al. 1997). In addition to providing major structural support to HDL particles, it also plays two important functional roles: the extraction of cholesterol from peripheral tissues by interacting with ABCA1; and in the activation of LCAT, which is a key factor in reverse cholesterol transport (Borhani et al. 1997). This section will cover all aspects of ApoA1, its life cycle, structure, variants and sensitivity to modifications.

1.3.1. PROTEINS OF HDL

The High Density Lipoprotein has been described to be cardio protective in nature, primarily owing to the positive effects exhibited by its various proteins (Heinecke 2010). In addition to the major protein ApoA1, other HDL proteins such as ApoA2, ApoD, ApoE, paraoxonase 1 (PON1), are also involved in various functions of HDL. Some of the key functions of the HDL enzymes are detailed in Table 1.3.

Table 1.3. HDL proteins and their major functions

S.No.	Protein	Function	Reference
1.	Apolipoprotein A-I (ApoA1)	Main structural protein, lipid binding, ABCA1 binding for cholesterol efflux, LCAT activation	(Borhani et al. 1997)
2.	Apolipoprotein A-II (ApoA2)	Enhances hepatic lipase activity	
3.	Apolipoprotein D (ApoD)	Associated with LCAT, progesterone binding	
4.	Apolipoprotein E (ApoE)	LDL-receptor binding	(Barbier et al. 2006, 2)
5.	Apolipoprotein M (ApoM)	Transport of Sphingosine-1 Phosphate	
6.	Serum Paraoxonase 1 (PON1)	Anti-oxidant and anti-inflammatory effects	(Heinecke 2010)

As is evident from Table 1.3, ApoA1 is a key effector of major protective functions of HDL, especially in reverse cholesterol transport (Fielding and Fielding 1995).

1.3.2. LIFE CYCLE OF ApoA1

The overall life cycle of ApoA1 is closely linked to that of the HDL molecule itself. ApoA1 molecules are produced by the liver or intestines, or are released from lipolysed VLDL and chylomicrons (von Eckardstein, Nofer, and Assmann 2001). These free ApoA1 molecules accept cholesterol from peripheral cells through ABCA1 to form discoidal HDL molecules. They move through the RCT as explained in section 1.2.3, and are eventually cleared in the liver at the end of the reverse cholesterol transport, wherein they are recycled (Fielding and Fielding 1995).

The ApoA1 protein is expressed as a single polypeptide chain of 243 amino acids, with a 24 amino acid signal peptide sequence for its secretion. Once in circulation, it self-associates into a 4-helix bundle linked via their hydrophobic faces to transport cholesterol, triglycerides and lipids (Lewis and Rader 2005).

1.3.3. STRUCTURE OF ApoA1

ApoA1 is a 28kDa monomer that is synthesised by the liver and intestine. The first crystal structure of ApoA1 suggested a horseshoe like shape, constituted almost entirely of amphipathic α-helix that is punctuated by kinks at regular intervals introduced by proline residues (Borhani et al. 1997). This amphipathic α-helix enables it to bind with lipids and cholesterol through its hydrophobic face and with the aqueous exterior with its polar face (Murphy 2013). A schematic ribbon structure of ApoA1 based on its crystal structure (PDB Accession Number 1AV1) is shown below in Fig. 1.5.

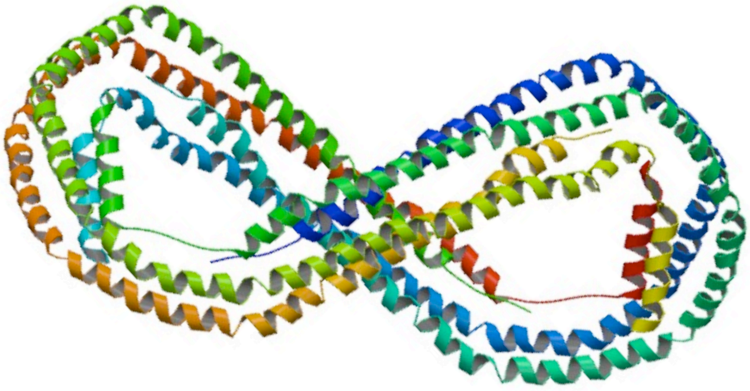


Figure 1.5. Ribbon structure of ApoA1 (PDB accession # 1AV1).

This structure was later confirmed by a subsequent study, revealing addition details. The structure of ApoA1 further showed a solvent-exposed loop from residues 159 to 180, which plays a key role in activation of LCAT (Wu et al. 2007).

1.3.4. ApoA1 VARIANTS

The ApoA1 gene has been well documented to have several polymorphisms: more than forty naturally occurring variants of ApoA1 have been reported in literature (Matsunaga et al. 2010). Some of the variants have been listed in Table 1.4.

Table 1.4. Variants of ApoA1

S.No.	ApoA1 Variant	Mutation	Physiological Consequence	Reference
1.	Milano	R173C	Reduced HDL but healthy cardiovascular system	(Weisgraber et al. 1983)
2.	Paris	R151C	Reduced HDL but healthy cardiovascular system	(Bruckert et al. 1997)
3.	Pisa	L141R	Absence of HDL cholesterol	(Miccoli et al. 1996)
4.	Finland	K159R	Hypoalphalipo-proteinemia	(Miettinen et al. 1997)
5.	Iowa	G026R	Amyloidosis	(Benson et al. 1991)
6.	Helsinky	K107Δ	Amyloidosis	(Ramella et al. 2012)
7.	Fukuoka	E110K		(Takada et al. 1990)

Of the above listed variants, two variants, Milano and Paris, are characterised by a single point variation of Arginine to Cysteine at 173 and 151 respectively (Klon et al. 2000). Both these variants have been reported in populations where people had a healthy cardiovascular system despite low HDL levels (Rocco et al. 2010). An important point to note is that wild type ApoA1 contains no cysteine residues, and hence these cysteine variants are hypothesised to be more stable due to formation of homodimers through disulphide bridges (Klon et al. 2000). Hence,

in this thesis work, special focus has been laid on the generation of these two ApoA1 cysteine variants, to enable their future applications in therapeutic areas.

1.3.5. MODIFICATIONS ON ApoA1

In addition to naturally occurring variations, ApoA1 is also highly susceptible to modifications under oxidising conditions. Myeloperoxidase (MPO), a defensive enzyme, is expressed by neutrophil granulocytes to fight bacterial infections (Klebanoff 2005). However, elevated levels of MPO have been reported in atherosclerotic plaques, leading to a number of modifications on HDL/ApoA1, thereby rendering them dysfunctional (Smith 2010). Table 1.5 lists some of the characterised modifications on ApoA1 by MPO, along with functional consequences that have been reported so far.

Table 1.5. MPO-mediated modifications of ApoA1 and its consequences

S.No	Type of Modification	Modified amino acid generated	Residues modified	Mechanism	Functional Consequences *in vitro*	Reference
1.	Chlorination of tyrosine	3-chloro tyrosine	Tyr-166 Tyr-192	MPO + H2O2 + Cl- ion generated (HOCl) hypochloite	Loss of Cholesterol acceptor activity	(Shao et al. 2005)
2.	Nitration of tyrosine	3-nitro tyrosine	Tyr-166 Tyr-192	MPO + H2O2 + NO2 generated (ONOO-) peroxynitrite	Loss of LCAT activation	(Shao et al. 2005)
3.	Oxidation of Methionine	Methionine sulfoxide	Met-86 Met-112 Met-148	MPO + H2O2 + Cl- ion generated HOCl hypochlorite	Loss of cholesterol acceptor activity Loss of LCAT activation	(Shao et al. 2008)
4.	Hydroxylation of tryptophan	Mono and Dihydroxy tryptophans	Trp-8 Trp-50 Trp-72 Trp-108	MPO + H2O2 + Cl- ion generated HOCl (hypochlorite)	Loss of Cholesterol acceptor activity	(Peng et al. 2008)
5.	Carbamylation of lysine		Lys-226	MPO + H2O2 + SCN- (thiocyanate)	Generate Proatherogenic and pro-inflammatory particles	(Brubaker et al. 2006)

Among the residues affected by MPO-oxidation, Tryptophan-modifications have been shown to be most consequential. Subsequently, an MPO-mediated oxidation-resistant mutant of ApoA1 was generated by substituting the four Trp residues with Phe residues (Peng et al. 2008).

Despite extensive studies on the effects of oxidation on wild type ApoA1, there is yet not conclusive data to showcase the behaviour of ApoA1 variants, especially Milano & Paris, under oxidative stress. It is hence of extreme interest to generate robust processes for the production of both wild type and cysteine variants of ApoA.

1.3.6. CLINICAL APPLICATIONS OF APOA1 – DIAGNOSTIC AND THERAPEUTIC

Recent studies, based on the sensitivity of ApoA1 to MPO-mediated modification, have established oxidised ApoA1 as a diagnostic target for the early detection of atherosclerosis. Antibodies specific to oxidised residues on the surface of ApoA1 are currently being explored for use in the early diagnosis of atherosclerosis (Nakano and Nagata 2003).

High levels of ApoA1 have been correlated with a decreased risk of atherosclerosis, and it has long since been hypothesised that elevating plasma ApoA1 levels could lead to regression of atherosclerosis (Fazio and Linton 2003). In addition to the above main function, ApoA1 has recently also been suggested to be used during in other therapeutic interventions like cancer therapy (Su et al. 2010). Keeping in mind the vast diagnostic and therapeutic applications of ApoA1, this thesis focuses on the development of an integrated process for the generation of wild type and variants of ApoA1 in a suitable recombinant protein-producing host.

1.4. EXPRESSION HOST

1.4.1. INTRODUCTION TO EXPRESSION SYSTEMS

The advent of recombinant DNA (rDNA) technology enabled the industrial production of heterologous proteins, making affordable therapeutics and

reducing the cost of several diagnostic and therapeutic interventions. Human insulin was the first heterologous protein produced in the laboratory in 1977 (Porro et al. 2011). Several expressions systems are currently being used for the expression of heterologous proteins: like bacterial prokaryotic expression systems (by far *E. coli*) and eukaryotic systems (yeast and mammalian expressions systems). In addition, plant systems and transgenic animals are being studied for the expression of recombinant therapeutics despite their difficulties, extreme high-cost and ethics involved in their production.

1.4.2. BACTERIAL EXPRESSION SYSTEMS

The bacterial expression system *Escherichia coli* is the most exploited system for the expression of recombinant proteins. *E. coli* is favoured as a host for the expression of heterologous proteins for a number of reasons: short doubling time, well established genome, ease of manipulation, GRAS (Generally Regarded As Safe) status, the ability to scale-up production to industrial scale at minimal costs (Makrides 1996). However, plasmid instability and the lack of complex post translational modification machinery like glycosylation limit their use (Porro et al. 2011).

1.4.3. EUKARYOTIC EXPRESSION SYSTEMS

The yeasts *Saccharomyces cerevisiae* and *Pichia pastoris* have been developed into highly successful systems for the expression of heterologous proteins (Cereghino and Cregg 2000). The issue of plasmid instability often faced with prokaryotic systems is overcome in eukaryotes due to stable integration of the gene of interest into the host genome, which is usually achieved through homologous recombination. The ability to grow yeasts in simple, defined media to high cell densities and their ability to perform post-translational modifications make them favourites for the expression of recombinant proteins (Demain and Vaishnav 2009). Nevertheless, hyper-mannosylation remains as a major drawback while working with proteins that require glycosylation. As the target protein in this work (ApoA1) has no glycosylation sites, this would not be affected by expressing in a lower eukaryotic host like yeast.

1.4.4. *Pichia pastoris* EXPRESSION SYSTEM

The *Pichia pastoris* expression system serves as a suitable system for the cost-effective production of value added proteins. Numerous heterologous proteins have been successfully expressed by the *Pichia Pastoris* expression system (Cereghino and Cregg 2000). The *P. pastoris* system is also generally regarded as being faster, easier, and less expensive to use than expression systems derived from higher eukaryotes such as insect and mammalian tissue culture cell systems and usually gives higher expression levels (Klein 1998; Sreekrishna et al. 1997). Overexpression of heterologous proteins in *P. pastoris* is achieved by the AOX1 promoter, which is activated under carbon starvation (Daly and Hearn 2005).

1.4.5. HETEROLOGOUS EXPRESSION OF PROTEINS IN *P. pastoris*: THE AOX1 SYSTEM

Pichia pastoris is one of approximately a dozen yeast species representing four different genera capable of metabolizing methanol. The methanol metabolic pathway appears to be the same in all yeasts and involves a unique set of pathway enzymes. The first step in the metabolism of methanol is the oxidation of methanol to formaldehyde in the peroxisome, generating hydrogen peroxide in the process, by the enzyme alcohol oxidase (AOX). The H_2O_2 produced by the AOX reaction is metabolised by catalase in the peroxisome (Fig. 1.6). AOX is strongly repressed by many alternate carbon sources such as glucose, glycerol and even ethanol, and is induced by carbon starvation when grown with methanol as the sole carbon source. Although alcohol oxidase is not specific for methanol and is capable of oxidizing other primary alcohols, the activity of the enzyme decreases as the number of carbons in the alcohol increases. The conversion of methanol to formaldehyde is the rate-limiting step in utilizing methanol, due to the poor affinity of AOX to O_2, and is regulated by increasing the amount of AOX enzyme in the cells (Daly and Hearn 2005).

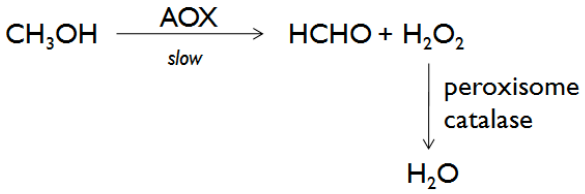


Figure 1.6. Alcohol oxidase system.

The alcohol oxidase enzyme is encoded for by two genes: AOX1 and AOX2. Most AOX in methanol-grown *Pichia pastoris* cells is coded for by the AOX1 gene (J. Cregg et al. 1985). Hence, transformation of gene of interest into the *Pichia* genome at the AOX1 gene provides the potential to overexpress heterologous proteins (J. Cregg et al. 1985).

As the *P. pastoris* expression vector does not contain a yeast origin of replication, it is essential for the integration of the expression cassette into the host genome. This recombination is possible through the AOX1 or aox1 loci at the 5'AOX1 promotor region. This homologous recombination could result in either the conservation of native AOX1 activity or disruption of AOX1 gene, resulting in Mut^{+} and Mut^{S} phenotypes, respectively (Fig. 1.7).

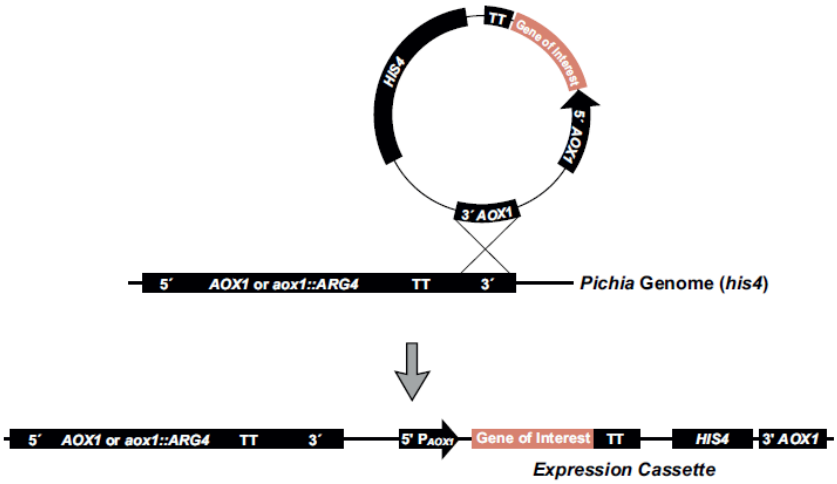


Figure 1.7(a) Generation of *Pichia pastoris* transformants of Mut^{+} phenotype.

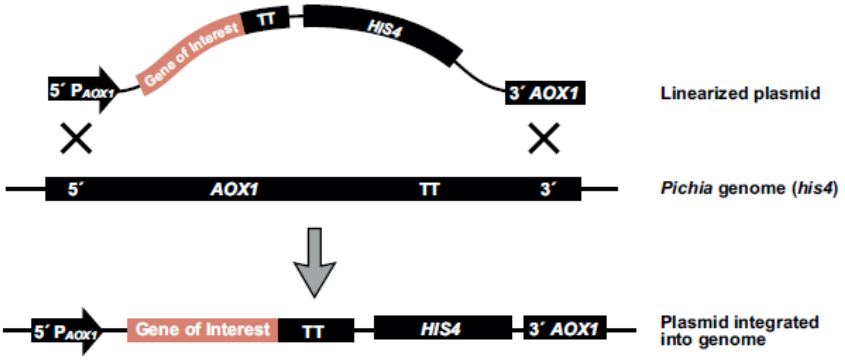


Figure 1.7(b) Generation of *Pichia pastoris* transformants of Mut^S phenotype.

Once the expression cassette of the target gene has integrated into the host genome, expression is achieved through carbon starvation and subsequent activation of the AOX1 promotor resulting in the expression of heterologous proteins.

1.5. GOAL AND BROAD OBJECTIVES

This thesis has focused on the development of an integrated process for the efficient generation of wild type and variants of human apolipoprotein A-I in *Pichia pastoris*.

The following objectives outline the research methodology that has been envisaged in this thesis work:

1. Cloning, expression of wild type rhApoA1 in *P. pastoris* X-33
2. Purification of wild type rhApoA1 in *P. pastoris* X-33
3. Scale-up of expression & purification of wild type rhApoA1 in *P. pastoris* X-33
4. Expression, purification of wild type rhApoA1 expressed in protease-deficient *P. pastoris* strain SMD-1168 and comparison with protein expressed with *P. pastoris* wild type strain X-33
5. Generation of rhApoA1 variants: Milano & Paris

Chapter 2

Generation of wild-type rhApoA1 in *P. pastoris*

2.1. INTRODUCTION TO CLONING IN *P. pastoris*

The methylotrophic yeast *Pichia pastoris* has long since been explored for the heterologous expression of proteins through the alcohol oxidase promoter (Cereghino and Cregg 2000). A number of *Pichia pastoris* expressed recombinant products like insulin and hepatitis-B vaccine have already hit the Indian market (Shekhar 2008). It is hence a very suitable host for the overexpression and production of recombinant ApoA1, as envisaged in this thesis.

Several expression vectors for *P. pastoris* are commercially available with Invitrogen, with most of them containing a unique multiple cloning site, resistance to the antibiotic Zeocin and signal peptides for secretory expression. The vector pPICZα is one of the widely used vectors containing all the above described features, and has been used in the present study too (James M. Cregg et al. 2009, 13). The functioning of AOX1 promoter has already been elucidated in section 1.4.5.

2.2. EXPERIMENTAL

2.2.1. MATERIALS

The construct containing human ApoA1 gene was purchased from Open Biosystems (EHS1001-5646018). Oligonucleotides for PCR amplification and cloning were custom-ordered from Sigma-Aldrich (Bangalore, India). All chemicals and reagents for expression and purification were from Sigma-Aldrich (Bangalore, India or Lyon, France) and BD Biosciences (Le Pont de Claix, France).

Expression studies in benchtop bioreactors were carried out on Sartorius-Stedim Biostat B-plus bioreactors monitored by MCSF Win data acquisition software (Sartorius), DNA sequencing was performed through Millegen (Labège, France).

The *P. pastoris* strain X-33 and vector pPICZαA were kind gifts from Prof. Rajan R. Dighe, Department of Molecular Reproduction, Development and Genetics (MRDG), Indian Institute of Science, Bangalore, India.

2.2.2. CLONING OF rhApoA1 GENE INTO pPICZαA

The sequence corresponding to human ApoA1 was amplified by Polymerase Chain Reaction using the primers 5'ATGAATTCGATGAACCCCCCCAGAGC3' and 5'ATGCGGCCGCTCACTGGGTGTTGAG3' to introduce *EcoRI* and *NotI* sites respectively to facilitate cloning. PCR was carried out using 35 cycles of denaturation (94°C for 30"), annealing (56°C for 30"), extension (72°C for 1'), followed by a final extension at 72°C for 10 mins. The reverse primer introduced a stop codon at the end of the gene to prevent expression of the optional hexa-histidine tag in the vector. The amplified fragment was cloned into the *Pichia pastoris* expression vector pPICZαA downstream of the AOX1 promoter and the resulting recombinant DNA construct (pPICZα-ApoA1) was verified by DNA sequencing using AOX1-specific primers by Millegen (Labège, France).

2.2.3. TRANSFORMATION INTO *P. pastoris*

The plasmid pPICZα-ApoA1 was linearized using *PmeI* and electroporated into competent *Pichia pastoris* X-33 cells, as per the manufacturer's instructions. To the transformed cells, 1ml ice-cold 1M sorbitol was added, and the cells were allowed to stand at 30°C for 30 mins, after which they were plated on YPDS plates containing 200µg/ml Zeocin™ for selection. The plates were incubated at 30°C for selection of transformants, and clones exhibiting high resistance to Zeocin™ (up to 2 mg/ml) were taken further for genomic DNA and subsequent expression analysis.

2.2.4. COLONY PCR ANALYSIS

In order to confirm the integration of the recombinant DNA construct pPICZα-ApoA1 into the Zeocin-resistant *P. pastoris* clones, Colony-PCR was performed on select transformed clones. The primers and PCR conditions were identical to that used to amplify the ApoA1 gene for cloning (Section 2.2.2). Following PCR, the various reactions were analysed by 0.8% agarose gel electrophoresis and stained with SYBR Safe stain.

2.2.5. EXPRESSION STUDIES ON FLASK CULTURES

Several clones of *Pichia pastoris* transformed with pPICZα-ApoA1 that exhibited high resistance to Zeocin were screened in shake flask for screening for high-levels of expression of rhApoA1.

From the YPD plate containing 2mg/mL Zeocin, five clones were selected for preliminary expression studies, along with a negative control (*Pichia pastoris* transformed with just the vector pPICZαA without insert). Single colonies of each clone were inoculated independently in 10ml BMGY containing 200 µg/ml Zeocin as a preculture. After overnight growth at 30°C and 200 rpm, the precultures were inoculated into 140 ml of fresh BMGY (buffered glycerol complex media). After about 24 hrs, when the cells had grown to a reasonable quantity (based on their OD at 600nm), they were centrifuged and resuspended in 150 ml of BMMY (buffered methanol complex media). Induction was carried out for a total of 5 days; with methanol being replenished every 24 hrs at 0.5%. At the end of the induction, comparative day-wise expression of select clones along with the control were analysed by SDS-PAGE and Western blotting.

2.2.6. EXPRESSION IN 2L BENCHTOP BIOREACTOR

Subsequent to preliminary expression studies on shake flasks, the best expressing clone was taken further for scale-up in a 2 l benchtop bioreactor.

First, a single colony was inoculated and grown in baffled flasks containing 100 ml of buffered glycerol-complex medium (BMGY, 100mM potassium phosphate buffer, pH 6.0, 13.4 g/L YNB, $4x10^{-4}$ g/L biotin, 10 g/L glycerol and 150 µg/mL Zeocin™) up to an OD (600nm) of 4-8, and this was inoculated in 2L of BMGY in a BIOSTAT® Bplus laboratory 2l bioreactor with a starting OD_{600} of about 0.3. The temperature and pH were maintained at 30°C and 6.0 respectively, and dissolved oxygen level was maintained at 15% saturation by regulating aeration and agitation in a cascade manner. After complete consumption of glycerol in the medium (verified by standard glycerol assay; data not shown), a methanol fed-batch phase was initiated by adding methanol every 12 hrs to a final concentration of 0.5%. Samples were drawn every 24 hrs to follow the growth

profile and protein production. After 120 hrs of induction, the culture was harvested and the cell-free broth was frozen at -80°C.

2.2.7. SDS-PAGE AND WESTERN BLOTTING

Protein samples were analysed by 12% SDS-PAGE, typically under reducing conditions with DTT. In order to probe for rhApoA1 (western blotting), the proteins from the acrylamide gel were transferred to a 0.45 µm nitrocellulose membrane using Transblot SD transfer apparatus (Bio-Rad, France) according to the manufacturer's instructions. The membrane was blocked with PBST containing 3% skimmed milk, and probed with goat anti-humanApoA1 antiserum (part of an ApoA1 immunoturbidometry-based detection kit, Apolipoprotein A1 FS, Cat. No. 17102, DiaSys, France) followed by rabbit anti-goat IgG conjugated to HRP (Jackson ImmunoResearch, PA, USA), and visualised by Opti-4CN colorimetric detection kit (Bio-Rad, France). In order to reduce the background generated by cross-reactivity of the antibodies in the primary antiserum (goat anti-humanApoA1) to native *P. pastoris* proteins, 100 µg of lyophilized *P. pastoris* control broth (without transformation of any vector) was added to the primary antiserum solution during blotting.

2.3. RESULTS

2.3.1. CLONING OF rhApoA1 GENE IN pPICZαA AND TRANSFORMATION INTO *P. pastoris*

The gene corresponding to ApoA1 was amplified using the primers as described in section 2.2.2. After amplification, the PCR product was cloned into a T-A cloning vector pXcmKn12, and subsequently restriction digested using *EcoRI* and *NotI* to sub-clone into the target vector pPICZαA. The resulting construct was transformed into competent *E. coli* DH5α cells and selected by resistance to 50µg/ml Zeocin. The construct was verified for correct integration of target gene by restriction digestion analysis and DNA sequencing using AOX1-specific primers. The sequencing data is analysed in Fig. 2.1, highlighting important features of the construct.

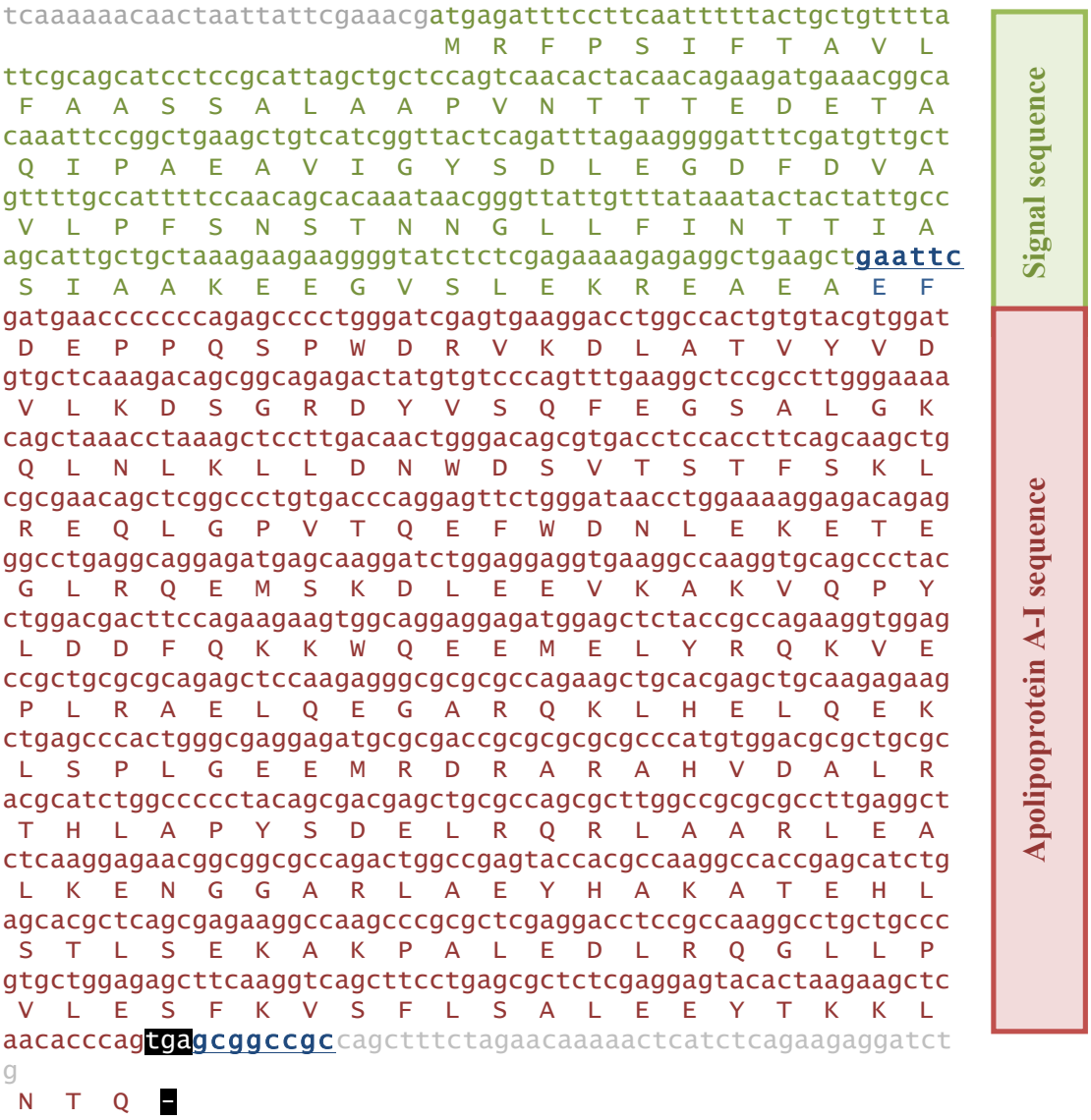


Figure 2.1. DNA sequencing results of pPICZα-ApoA1 highlighting the α-mating factor signal sequence, cloning sites (*EcoRI* and *NotI*) and Apolipoprotein A-I gene.

The resulting construct, pPICZα-ApoA1 (Fig. 2.2) contained an upstream α-mating factor signal sequence for secretion of the expressed recombinant protein followed by the gene of interest. It also contained a resistance gene to the antibiotic Zeocin, which would act as a selection marker upon transformation of this construct. An important feature to note is the incorporation of a stop codon in the reverse primer to exclude the optional c-myc epitope and hexahistidine tag present in the vector. This has been done in order to express the protein as close as possible to its native state, to maintain maximum activity. This also resulted in minimal inclusion of additional amino acids, thereby eliminating the need for additional digestion steps during downstream processing.

After verification by DNA sequencing, the construct was linearised by digestion with the restriction enzyme *PmeI*, and subsequently electroporated into competent *P. pastoris* X-33 cells as described in the methods section. Subsequently, transformants were sequentially patched on to YPD agar plates containing increasing concentrations of Zeocin up to 2 mg/ml (Fig. 2.3).

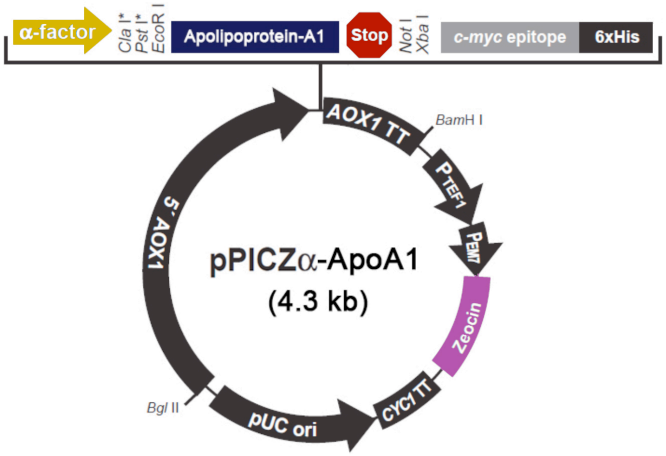


Figure 2.2. Schematic representation of pPICZα-ApoA1 construct.

In addition to tolerance to 2 mg/ml Zeocin, all positive clones were also checked for the integration of the complete ApoA1 gene into the host genome. Colony PCR was performed on all clones resistant to 2 mg/ml Zeocin using ApoA1-specific primers under the conditions as detailed in the experimental section. Subsequently, all reaction mixtures were subjected to 0.8% agarose gel electrophoresis (Fig. 2.4), which clearly confirmed the presence of ApoA1 gene in the transformed *P. pastoris* genome.

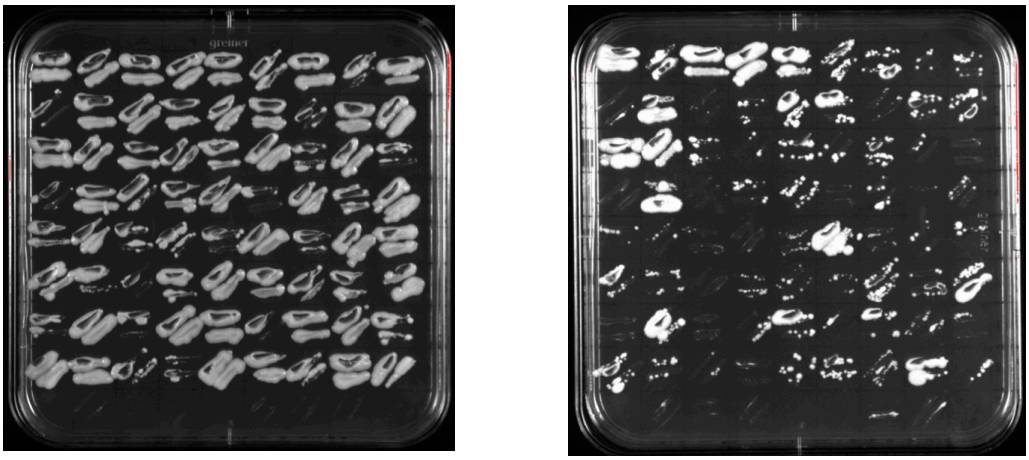


Figure 2.3. Screening of patched colonies growing on (a) 1 mg/mL and (b) 2 mg/mL concentration of Zeocin. After patching, the plates were incubated at 30°C for about 48 hrs to check for growth.

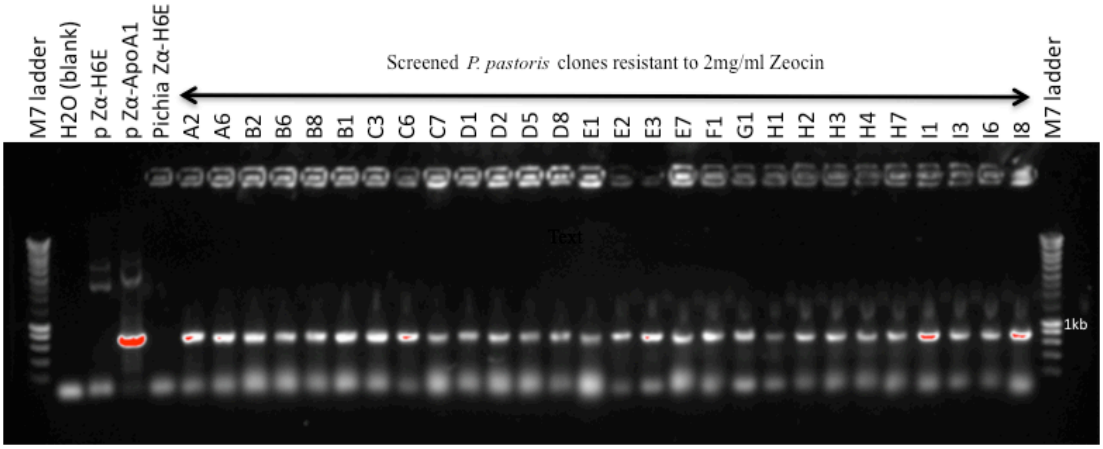


Figure 2.4. Colony PCR analysis of control (H_2O), plasmids of vector (pZα-H6E) and vector containing ApoA1 gene (pZα-ApoA1), and *P. pastoris* transformed with vector (Pichia Zα-H6E) and *P. pastoris* clones from the YPD-agar containing 2 mg/ml Zeocin (from Fig. 2.3(b)). M7 ladder (SmartLetter MW-1700-10) was from Eurogentec (France).

Five of the clones that exhibited maximum resistance to Zeocin and verified to have the gene incorporated into the genome were further studied for preliminary expression in shake flasks.

2.3.2. EXPRESSION OF rhApoA1 IN *P. pastoris*

Five clones that exhibited high resistance to Zeocin (2 mg/ml) were independently inoculated into BMGY media for expression studies. Induction was carried out as explained in the methods section.

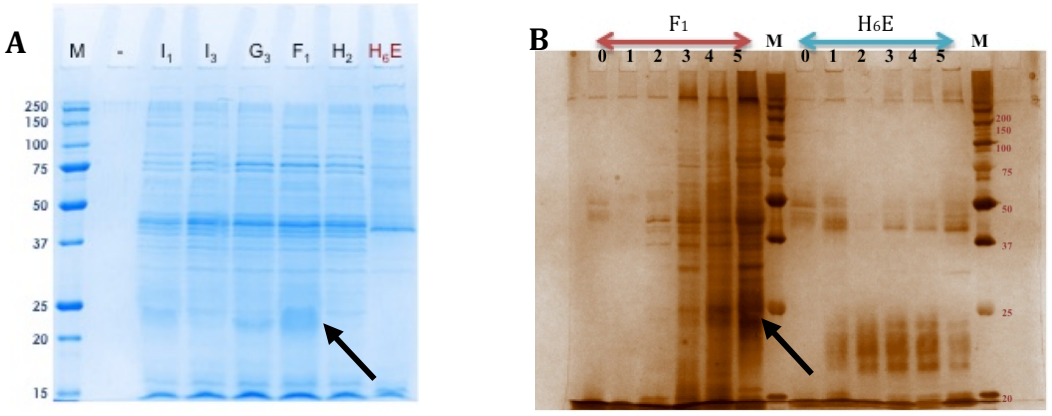


Figure 2.5. Comparative rhApoA1 expression analysis by SDS-PAGE of (a) all clones with control upon harvesting (stained with CBB); and (b) day-wise expression profiles of clones F1 and control (stained by Silver staining).

As evident in figure 2.5(a), all screened clones expressed rhApoA1, and the intensity of expression increased with the duration of induction (Fig. 2.5(b)). The negative control (*P. pastoris* X-33 transformed with the pPICZαA vector) showed no expression of rhApoA1, as expected. Upon the conclusion of this preliminary study, the clone with highest rhApoA1 expression level (clone ID F_1) was taken up for further scale-up studies.

2.3.3. EXPRESSION OF rhApoA1 IN *P. pastoris* (2 L BIOREACTOR)

After preliminary studies on the expression of rhApoA1 in shake flasks, production was carried out in a 2 l benchtop bioreactor. The parameters were maintained as explained in Section 2.2.6, yielding the profile in Fig. 2.6. The temperature and pH were maintained in real time by various controllers, and cell-growth and substrate metabolism was implied out of the dissolved oxygen levels maintained by aeration and agitation systems.

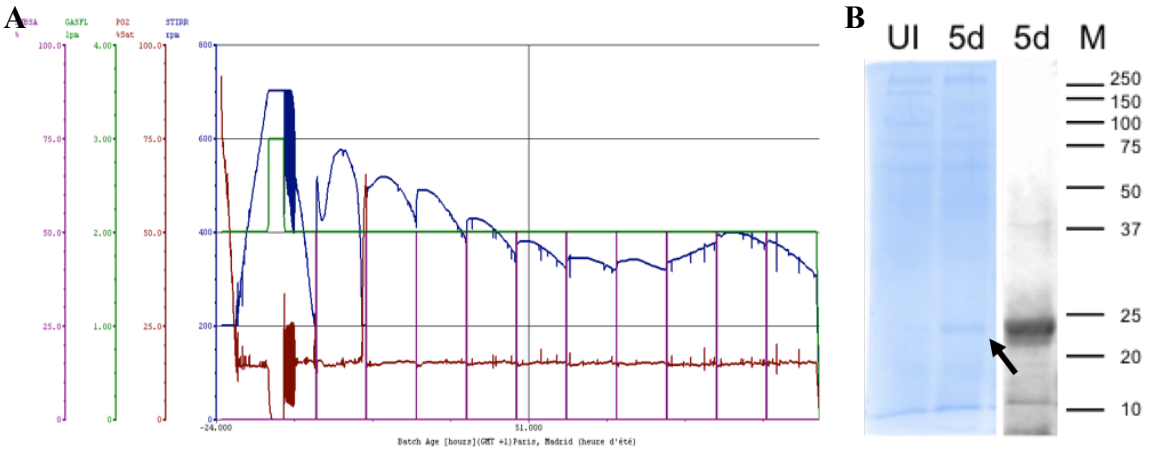


Figure 2.6. (a) Profile and (b) SDS-PAGE & Western Blotting analysis of rhApoA1 expression in *P. pastoris* X-33.

In the glycerol batch phase (first 24 hrs), there was a consistent drop in pO_2 levels (red) until it reached set point (15%), subsequently, the agitation (blue) and aeration (green) increased to maintain the pO_2 levels. After exhaustion of substrate, the agitation decreased until set low-limit (200 rpm), in time for the commencement of induction with methanol (pink spikes). With every methanol addition episode (every 12 hrs), there was a corresponding increase in agitation indicating consistent metabolism. At the end of the production, the expression levels were verified by SDS-PAGE and western blotting (Fig. 2.6(b)) prior to

induction (UI) and after 5-days of induction with 0.5% methanol (5d), and the expression in the bioreactor was successfully confirmed.

2.4. CONCLUSION

This chapter highlights the cloning of wild type ApoA1 gene in the expression vector pPICZαA and the successful expression of rhApoA1 in *Pichia pastoris*. Reproducible and consistent expression levels were observed in shake-flasks cultures over two batches, paving way for preliminary data for developing a bioprocess for production of rhApoA1 in *P. pastoris*. Subsequently, the production was also reproduced under chemostat conditions (temperature, pH, pO_2 levels maintained), and the expression was verifiable by standard biochemical methods (SDS-PAGE and Western Blotting).

A number of expression systems have been explored for the generation of recombinant ApoA1. The bacterial expression host *E. coli* has been previously demonstrated for rhApoA1 production (Panagotopulos et al. 2002), but low expression levels even after codon optimisation (Ryan, Forte, and Oda 2003) and intracellular expression have been limiting factors in scaling up production. *P. pastoris* is an established system for the heterologous expression of proteins, in producing several of them at gram per litre levels (Klein 1998). The ability of *P. pastoris* to secrete proteins into the media helps reduce the complexity in downstream processing (Brake et al. 1984), especially when the target protein is being expressed in its near-native state without any additional affinity tags.

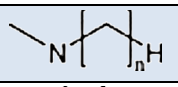
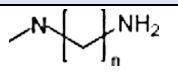
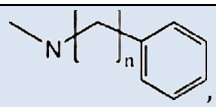
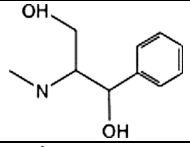
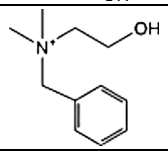
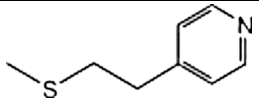
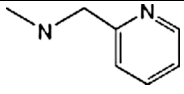
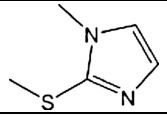
Chapter 3

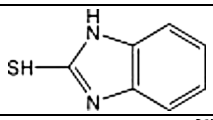
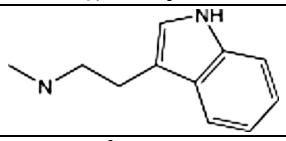
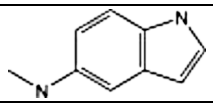
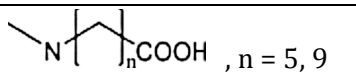
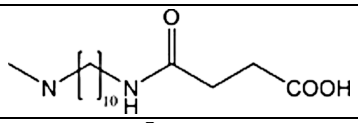
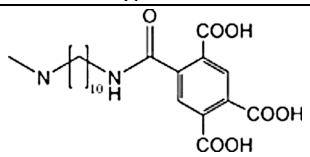
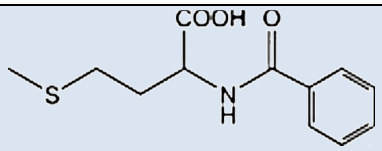
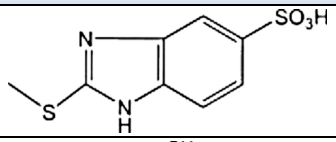
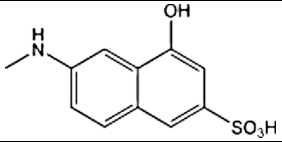
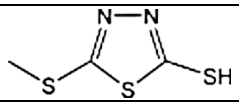
Purification of wild-type rhApoA1 expressed in *P. pastoris* by mixed-mode chromatography

3.1. INTRODUCTION TO MIXED-MODE CHROMATOGRAPHY

Mixed-mode chromatography or multi-modal chromatography has been developed for the rational use of multiple interactions in a controlled manner, in contrast to non-specific interactions (Pezzini et al. 2015). Mixed-mode ligands have been in existence for a long time, with hydroxyapatite chromatography, a combination of ionic and metal-affinity interactions, possibly making it the oldest form of mixed-mode chromatography (Gorbunoff and Timasheff 1984). Several other chromatographic methods, like dye-affinity, Histidine ligand affinity, peptide-affinity chromatography could also be classified as mixed-mode and have been well characterised (Pezzini et al. 2014). Several mixed-mode ligands are available in the market, and some of them have been listed below in table 3.1.

Table 3.1. Ligands for mixed-mode chromatography (Zhao, Dong, and Sun 2009). The ligands evaluated in this study have been highlighted in blue.

S.No.	Name	Structure	pKa
	Positively charged ligands		
1.	Alkylamine (n=6: *HEA Hypercel, Pall Life Sciences*)	 , *n* = 1–5, 6, 8	≈10
2.	α,ω-Diamino alkane	 , *n* = 2–6, 8, 10	≈10
3.	Phenylalkylamine (n=3: *PPA Hypercel, Pall Life Sciences*)	 , *n* = 1, 3, 4	6–7
4.	2-Amino-1-phenyl-1,3-propanediol		9.0
5.	N-Benzyl-N-methyl ethanol amine (*Capto™ adhere, GE Healthcare*)		–
6.	4-Mercapto-ethylpyridine (*MEP Hypercel, Pall Life Sciences*)		4.85
7.	2-Amino-methylpyridine		pKa_1 = 2.2, pKa_2 = 8.5
8.	Mercaptomethyl-imidazole		5.3

S.No.	Name	Structure	pKa
9.	2-Mercapto-benzimidazole		4.1
10.	Tryptamine		10.3
11.	5-Aminoindole		3.9
	Negatively charged ligands		
12.	Aminoalkyl carboxyl acid	 , n = 5, 9	5.2
13.	N-(3-Carboxypropionyl) aminodecyl amine		9.1
14.	N-pyromellityl aminodecyl amine		pKa_1 = 2.2, pKa_2 = 3.4, pKa_3 = 5.4
15.	2-Benzamido-4-mercaptobutanoic acid (Capto™ MMC and Streamline Direct CST I, GE Healthcare)		3.3
16.	2-Mercapto-5-benzimidazole sulfonic acid (MBI Hypercel, Pall Life Sciences)		–
17.	6-Amino-4-hydroxy-2-naphtalene sulfonic acid		–
18.	2,5-Dimercapto-1,3,4-thiadiazole		6.3

In order to purify rhApoA1 for potential therapeutic application, it was necessary to device a purification process that would specifically capture rhApoA1 from the *P. pastoris* expression broth in minimal steps. In an attempt to reduce the number of processing steps, the ApoA1 gene was already cloned in a manner that would eliminate the optional hexahistidine tag present in the pPICZαA vector, so that the expressed protein (chapter 2) could be obtained in its near-native form, without the addition of any affinity tags.

Previous reports on the overexpression of rhApoA1 in bacteria have all utilized a hexahistidine tag (Bergeron et al. 1997; Panagotopulos et al. 2002; Ryan, Forte, and Oda 2003), which was avoided in this work. From other sources (plasma, *P. pastoris*), a few methods have been documented that could prove useful for the capture of ApoA1 (plasma or recombinant) in its native-state (Feng et al. 2006; Marco Aurélio Zezzi Arruda et al. 2011). These methods have also been investigated for the recovery of the expressed rhApoA1 from *P. pastoris* expression broth.

In this chapter, novel mixed-mode chromatography methods have been developed to recover rhApoA1 from *P. pastoris* expression broth. The defined mixed-mode ligands HEA HyperCel, PPA HyperCel (Pall Life Sciences) and CaptoMMC (GE Healthcare) have been evaluated for their ability to capture rhApoA1 from *P. pastoris* expression broth (highlighted in rows 1, 3 and 15, respectively). These mixed-mode sorbents have been extensively characterised in our laboratories (Ranjini et al. 2010; Pezzini et al. 2014; Pezzini et al. 2015), and hence were explored conditions for the capture of rhApoA1 from the *P. pastoris* expression broth.

Mixed mode ligands offer multiple-types of interactions, such as ionic, hydrophobic hydrogen bonding, and the balance between these interactions allow new selectivity (Zhao, Dong, and Sun 2009). The salt tolerance afforded by the hydrophobic character reduces the need for sample dilution; and is the key reason for choosing mixed mode chromatography for purifying rhApoA1. Below 20mS/cm, the primary mode of interaction is electrostatic and the mixed mode ligands used in this study have cation exchanger groups allowing an extended pH binding range compared to traditional ion exchangers which permit protein adsorption only near the pI of the target protein.

In addition, the efficiency of these mixed-mode ligands in the efficient capture of rhApoA1 have been compared with earlier published methods as described in this section.

3.2. EXPERIMENTAL

3.2.1. MATERIALS

All chromatographic experiments were performed on AKTA chromatographic platforms (AKTA Purifier, AKTA Explorer, AKTA FPLC, GE Healthcare) and operated by Unicorn software (GE Healthcare). Prepacked PRC HEA Hypercel and PRC PPA Hypercel columns were from Pall Life Sciences (France), and prepacked HiTrap Capto MMC, HiPrep 26/10, Source 15Q and Resource 15RPC were from GE Healthcare (Uppsala Sweden).

All buffers and other reagents were prepared using analytical grade chemicals from Sigma Aldrich (Bangalore, India).

3.2.2. EXPERIMENTAL CONDITIONS FOR DERIVED PURIFICATION METHODS

3.2.2.1. PURIFICATION OF rhApoA1 BY CLOUD-POINT EXTRACTION

A cloud-point extraction protocol was derived from the method previously published for recovering plasma ApoA1 (Marco Aurélio Zezzi Arruda et al. 2011). For preliminary optimisation experiments, various concentrations of NaCl and Triton X-114 were added to 2 ml of *P. pastoris* expression broth containing ApoA1 at 4°C. The mixture was vortexed and allowed to stand for 5 mins at room temperature, until it separated into a dense surfactant-poor and a light surfactant-rich phase. The solution was then centrifuged at 1780g for 10 minutes to clearly separate out the two phases, following which the surfactant rich phase was transferred to a fresh tube. The surfactant rich phase was precipitated with cold-acetone to eliminate the detergent and the surfactant poor phase was precipitated with TCA followed by cold-acetone washes to eliminate the salts. All fractions were analysed over SDS-PAGE analysis for the presence of rhApoA1, and proteins in the surfactant rich & poor phases were estimated by Bradford's assay and analysed by 12% SDS-PAGE for verifying partitioning. Subsequently, under optimised conditions, the strategy was scaled-up for a larger volume (50 ml) of *P. pastoris* broth.

3.2.2.2. PURIFICATION OF rhApoA1 BY COLD-ACETONE PRECIPITATION

Purification of rhApoA1 from *P. pastoris* expression broth was carried out as described by Feng et al (2006). Subsequent to expression of rhApoA1, the *P. pastoris* culture was centrifuged at 4,000 rpm for 10 mins to separate the cells from the supernatant. Cold acetone (-20°C) was added to 20% final concentration and incubated at -10°C for 5hrs. After the incubation period, the mixture was again centrifuged at 12,000 rpm for 20 mins after which the acetone concentration in the supernatant was raised to 40%. Subsequently, the mixture was again incubated at -20°C for 5hrs, after which the mixture was centrifuged again. The pH of the supernatant was adjusted to 5.8, after which cold acetone was again added to 60% and centrifuged. The pellet containing rhApoA1 was resuspended in phosphate buffer (pH 7.4), desalted using a HiTrap Desalting column (i.d. 1.6 cm x 2.5 cm, GE Healthcare) to remove any residual salt, and subjected to ion exchange chromatography using quaternary amine (Source 15Q, i.d. 0.5 cm x 9.5 cm, GE Healthcare), in phosphate buffer at pH 7.4, and eluted using a salt gradient from 0 to 0.5 M NaCl, followed by a step to 1 M NaCl.

3.2.2.3. PURIFICATION OF rhApoA1 BY ION-EXCHANGE CHROMATOGRAPHY

As an improvisation of the published method in section 3.2.2.2 (Feng et al. 2006), the *P. pastoris* expression broth containing rhApoA1 was desalted and directly subjected to ion exchange chromatography to evaluate if the cold-acetone precipitation step could be eliminated.

In the first step, the *P. pastoris* expression broth was desalted using a HiPrep 26/10 column (i.d. 2.6 cm x 10 cm, GE Healthcare) with 50mM Tris, pH 8.0 at a flow rate of 7 ml/min. A method was programmed to process 10 ml of *P. pastoris* expression broth in a batch, yielding a desalted fraction of 17 ml volume. Five batches were performed and the eluted fractions were pooled for the subsequent ion exchange experiment.

Following desalting, the pooled fractions (~70 ml) were injected on to a pH 8.0 equilibrated Source 15Q column (i.d. 0.5 cm x 9.5 cm, GE Healthcare). After

collecting the non-retained fraction, the bound proteins were eluted in steps by using 100 mM, 200 mM and 1 M NaCl.

Finally, the fractions eluted with 200 mM NaCl were further subjected to reverse phase chromatography on a Source 15RPC column (i.d. 0.64 cm x 10 cm, GE Healthcare). The column was equilibrated water + 0.1% TFA (buffer A), and the following gradient was applied with acetonitrile + 0.085% TFA (buffer B): 0-15%B over 20CV; 15-25%B over 20CV; hold at 30%B for 5CV; hold at 80%B for 5CV; and hold at 0%B for 5CV. Peak fractions were collected and analysed by SDS-PAGE.

3.2.3. CHROMATOGRAPHIC CONDITIONS FOR NOVEL PURIFICATION METHODS USING MIXED-MODE CHROMATOGRAPHY

In addition to the above-explored published methods, several mixed-mode chromatography methods have been investigated and optimised for the recovery of rhApoA1 from *P. pastoris* expression broth.

3.2.3.1. PURIFICATION OF rhApoA1 BY HEA HYPERCEL

Prepacked PRC HEA Hypercel column (i.d. 0.8 cm x 10 cm) was first equilibrated with 50 mM Tris buffer, pH 7.4, 250 mM NaCl (binding buffer). Salt was added to give the buffer the same conductivity as the *P. pastoris* expression broth. The pH of the load was raised to 7.4 using 10 N KOH. After the column was equilibrated, the sample was injected, and non-retained fractions were collected. After the absorbance reached baseline the salt in the buffer was eliminated, through a linear gradient with 50 mM Tris, pH 7.4 over 20 column volumes. Elution was then carried out in steps using 50mM sodium acetate buffers at pHs 6.0, 5.0, 4.0 and 3.0. The eluted fractions were analysed by SDS-PAGE and western blots to verify the purification of rhApoA1.

3.2.3.2. PURIFICATION OF rhApoA1 BY PPA HYPERCEL

Pre-packed PRC PPA Hypercel column (i.d. 0.8 cm x 10 cm) was first equilibrated with 50 mM Tris buffer, pH 7.4, 250 mM NaCl (binding buffer). Salt was added to give the buffer the same conductivity as the *P. pastoris* expression broth. The pH

of the load was raised to 7.4 using 10 N KOH. After the column was equilibrated, the sample was injected, and non-retained fractions were collected. After the absorbance reached baseline the salt in the buffer was eliminated, through a linear gradient with 50 mM Tris, pH 7.4 over 20 column volumes. Elution was then carried out in steps using 50mM sodium acetate buffers at pHs 6.0, 5.0, 4.0 and 3.0. The eluted fractions were analysed by SDS-PAGE and western blots to verify the purification of rhApoA1.

3.2.3.3. PURIFICATION OF rhApoA1 BY CAPTO MMC

The Capto MMC column was first equilibrated with 50 mM acetate buffer, pH 5.0, 250 mM NaCl (binding buffer). The pH of the *P. pastoris* expression broth at the end of the fermentation batch was adjusted to pH 5.0 and injected on to the column. The non-retained flowthrough fraction was collected, and once the absorbance reached baseline, the salt was eliminated over a linear gradient using 50mM acetate buffer, pH 5.0 over 20 column volumes. Elution was then carried out in steps using 50 mM phosphate buffer at pH 7.0 and 50 mM Tris buffer at pH 8.5. The eluted fractions were analysed by SDS-PAGE and western blots to verify the successful purification of the target protein (rhApoA1).

3.2.4. VALIDATION OF PURIFIED rhApoA1: MASS SPECTROMETRY

Structural characterisation of the purified rhApoA1 was carried out over tandem mass spectrometry using an Agilent G6540A LC ESI-Q-TOF MS/MS. The purified elution fractions were individually lyophilized and reconstituted in 100µl of 6 M Urea, 50 mM Tris-HCl, pH 8.0. The proteins were reduced by adding 5 µl of 200 mM DTT in Tris, pH 8.0 and incubating the mixture at room temperature for 1 hour, and subsequently alkylated by adding 20 µl of 200 mM Iodoacetamide and incubating at room temperature (dark) for 1 hour. After alkylation, the protein mixture was diluted with Tris, and incubated with Trypsin to a final w/w ratio of 1:50 (trypsin:protein) and incubated at 37°C for 16-20 hrs. After trypsinisation, the pH was reduced with formic acid and injected into the Agilent G6540A LC ESI-Q-TOF MS/MS. The data obtained were analysed using the Agilent Mass Hunter software and matched with the ApoA1 sequence.

3.3. RESULTS

3.3.1. PURIFICATION OF rhApoA1 BY METHODS BASED ON PUBLISHED LITERATURE

3.3.1.1. PURIFICATION OF rhApoA1 BY CLOUD POINT EXTRACTION

Aliquots of *P. pastoris* expression broth (2 ml each) were treated with various concentrations of Triton X-114 (2% to 20%) and NaCl (0% to 15%). After incubation at room temperature and centrifugation, the upper surfactant rich phase (~ 200 µl) and the lower surfactant poor phase (~1800 µl) were separated and quantified for their protein content. The partition coefficient was plotted as a function of concentration of Triton X-114 added for various concentrations of salt (Fig. 3.1(a)), and all fractions were analysed by 12% SDS-PAGE analysis (Fig. 3.1(b)).

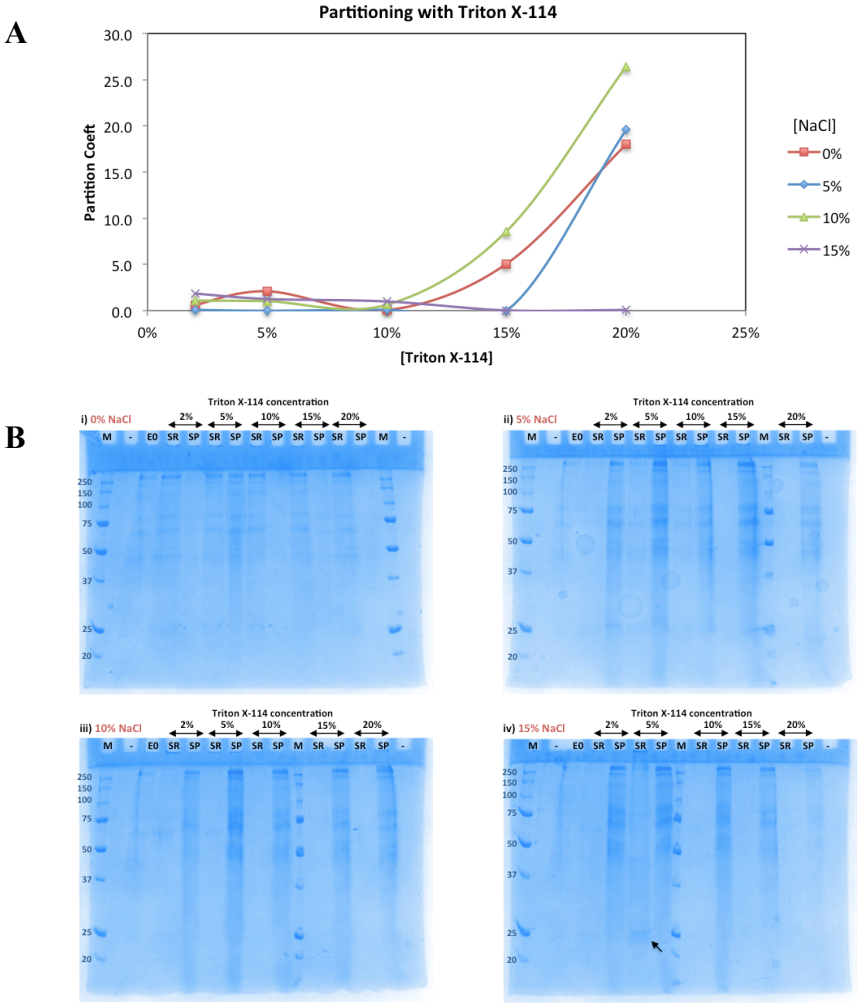


Figure 3.1. Cloud Point Extraction of rhApoA1 from *P. pastoris* expression broth under various [Triton X-114] & [NaCl]: (a) partition coefficient and (b) 12% SDS-PAGE.

After testing various concentrations of Triton X-114 and NaCl (Fig 3.1(a)), optimum recovery of rhApoA1 was achieved at 15% NaCl and 5% Triton X-114 (Fig. 3.1(b) *panel iv*, indicated by arrow). The combination of NaCl as electrolyte along with the non-ionic surfactant Triton X-114 has been shown to be effective in partitioning hydrophobic proteins from several sources (Lopes et al. 2007). In this case, the addition of NaCl helped achieve partitioning even at relatively lower concentrations (5% v/v) of Triton X-114.

After optimising conditions, the extraction method was scaled up to process 50 ml of *P. pastoris* broth containing rhApoA1. As evident in Fig 3.2(a), the solution clearly partitioned into surfactant rich (SR) & surfactant poor (SP) phases at room temperature. Furthermore, processing of aliquots of the SR & SP phases followed by their SDS-PAGE analysis revealed reproducibility and direct scalability of the optimised conditions (Fig 3.2(b)).

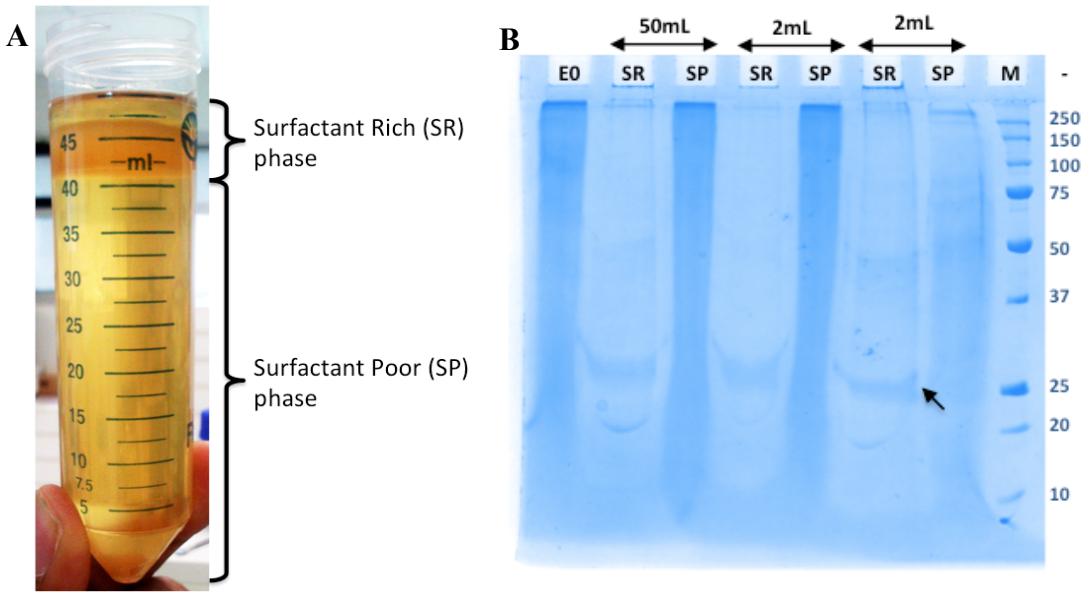


Figure 3.2. Cloud Point Extraction of rhApoA1 from 50ml of *P. pastoris* expression broth: (a) Partitioning and (b) 12% SDS-PAGE analysis

Despite the consistent reproducibility, eliminating the detergent from the surfactant rich phase was very difficult: dialysis, detergent exchange with CHAPS, on-column washing were attempted, but were unsuccessful. Washing with cold acetone was the only solution that eliminated the detergent (not completely) enough to analyse over electrophoresis. Hence, this process was

clearly not suitable for subsequent scaling-up to larger volumes and potential industrial exploitation.

3.3.1.2. PURIFICATION OF rhApoA1 BY COLD-ACETONE PRECIPITATION

Based on (Feng et al. 2006), capture of rhApoA1 was attempted by cold-acetone precipitation, followed by an ion-exchange chromatography polishing step. This is the only existing purification report available for the recovery of rhApoA1 expressed in *P. pastoris*. After three precipitation steps with cold acetone (-10°C) for 5 h each, followed by isoelectric precipitation and polishing by anion exchange chromatography, rhApoA1 was successfully recovered from the *P. pastoris* expression broth (Fig. 3.3(b)).

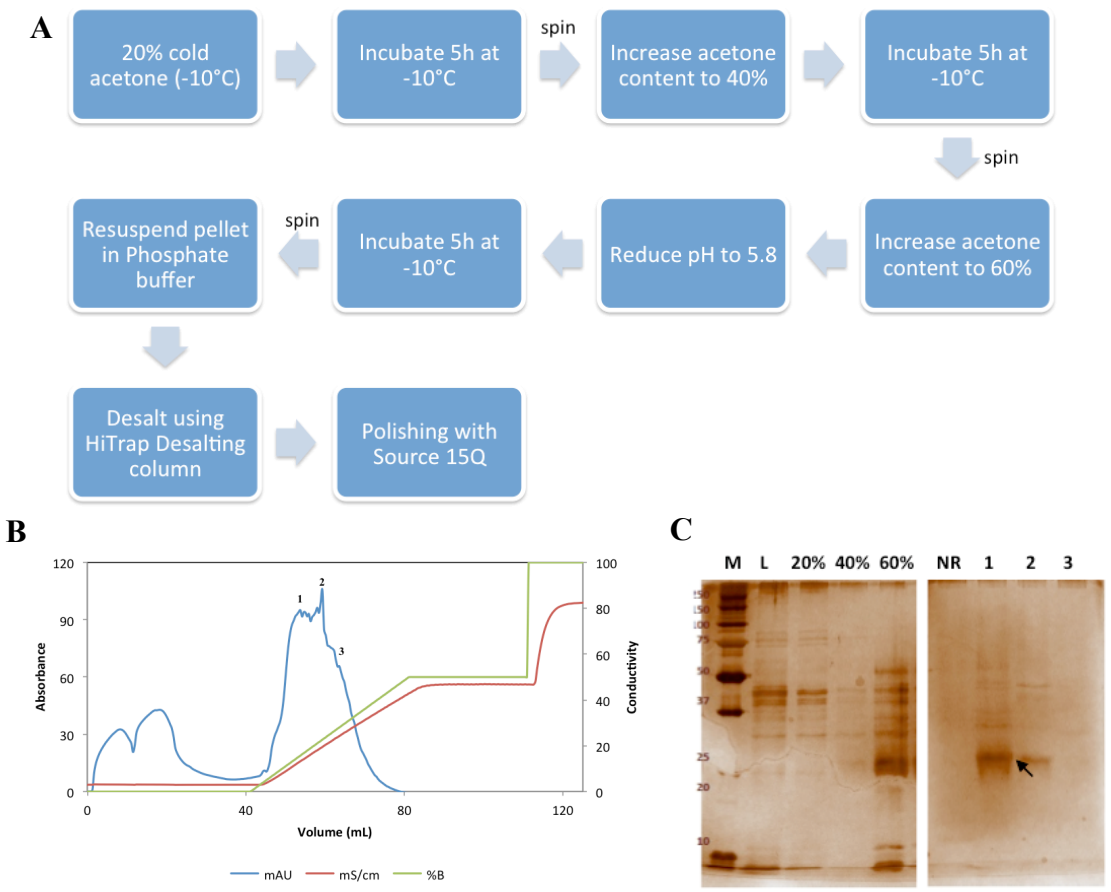


Figure 3.3. Purification of rhApoA1 by cold-acetone precipitation followed by ion exchange chromatography: (a) schematic of the method followed, (b) chromatogram of IEX over Source 15Q column and (c) SDS-PAGE analysis of various fractions.

Precipitation with cold acetone was highly effective in concentrating rhApoA1, which proved to be a useful precursor step to final polishing using anion exchange chromatography. Subsequent to the ion-exchange step, rhApoA1 was recovered with greater than 85% purity, with a few other additional higher molecular weight bands being present. Though this process was reproducible, utilisation of high concentrations of cold acetone (~ 60% acetone in the third precipitation step) coupled with extremely lengthy incubation periods (3 x 5 hrs) at sub-zero temperatures (-10°C) made this process unfeasible for scaling-up.

3.3.1.3. PURIFICATION OF rhApoA1 BY ION-EXCHANGE CHROMATOGRAPHY

As an improvisation to the method detailed above (Section 3.3.1.2), ion-exchange chromatography was applied directly to desalted *P. pastoris* expression broth containing rhApoA1. The purpose of this improvisation was to evaluate if the cold acetone treatment could be avoided, thereby reducing the processing time.

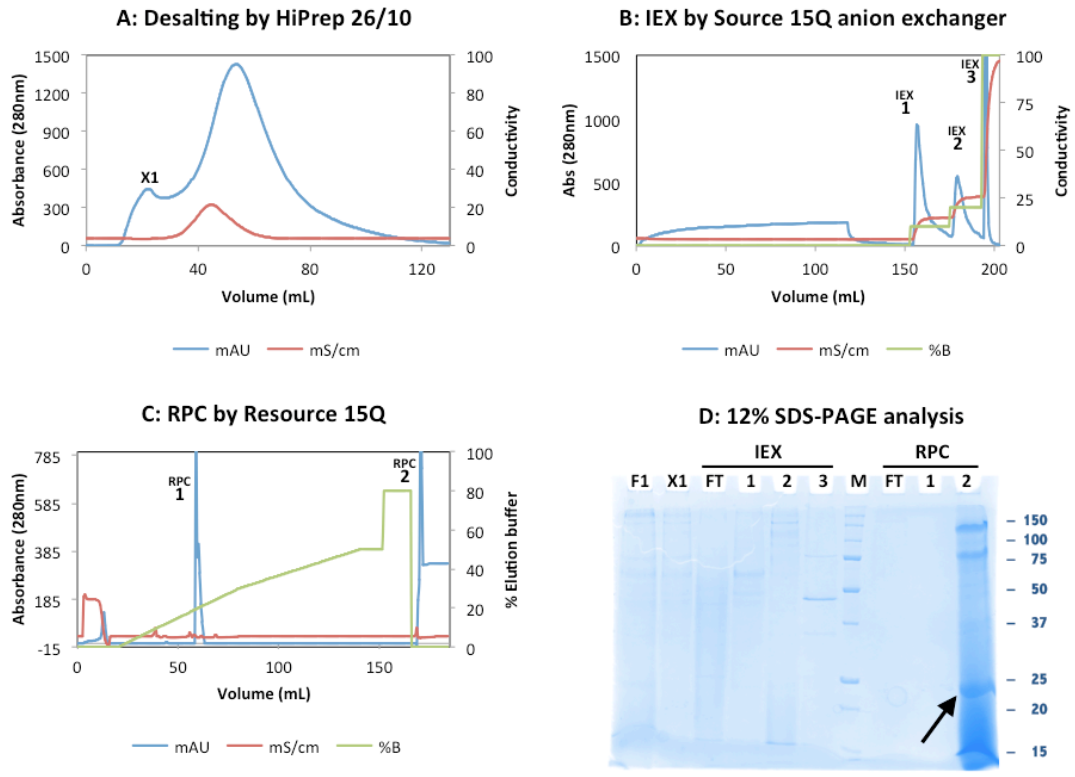


Figure 3.4. Purification of rhApoA1 by ion exchange chromatography. The *P. pastoris* expression broth containing rhApoA1 was first desalted (a), yielding a salt-free fraction (X1), which was further subjected to anion exchange chromatography (b). The elution fraction with 200 mM NaCl (IEX #2) was further polished by reverse phase chromatography (c), to recover rhApoA1 as verified by 12% SDS-PAGE analysis (d).

In the first step, the *P. pastoris* expression broth was desalted to remove the otherwise high conductivity contributed by ammonium sulphate present in the medium (BMGY/ BMMY). After initial optimisation, a method was programmed to efficiently desalt *P. pastoris* expression broth containing rhApoA1 (fraction X1) in batches of 10ml, yielding a typical profile as in Fig. 3.4(a).

This desalted *P. pastoris* broth from 5 chromatographic runs were then pooled and subjected to ion-exchange chromatography on a packed Source 15Q column as described in the experimental section. After initial optimisation, elution was achieved in two steps using 100 mM and 200 mM NaCl yielding the Chromatogram as in Fig. 3.4(b). The fraction eluted at 200 mM NaCl (peak IEX #2) was further subjected to polishing by reverse-phase chromatography (Fig 3.4(c)) to finally purify rhApoA1.

All fractions from various steps were analysed by 12% SDS-PAGE, which revealed the successful purification of rhApoA1 (Fig 3.4(d)). The purified fraction (RPC #2) was also verified by western blotting using polyclonal anti-ApoA1 antibody. The eluted fraction also showed aggregates of rhApoA1, consistent with previously published literature (Vitello and Scanu 1976). This method successfully circumvented the acetone precipitation steps, and we were able to recover rhApoA1 with an overall recovery of 33%. However, long processing times in desalting and limited flow-rates and difficulties in reproducibility of the RPC experiment were limiting factors in scaling this purification scheme.

3.3.2. PURIFICATION OF rhApoA1 BY MIXED-MODE CHROMATOGRAPHY

Despite a number of functional purification methods that were evaluated and improvised in the previous section, each technique showed severe limitations in their suitability for scale-up. It was hence imperative to look for novel purification approaches for efficiently capturing rhApoA1 from the *P. pastoris* expression broth. In this section, three defined mixed-mode ligands, HEA

HyperCel (Pall Life Sciences), PPA HyperCel (Pall Life Sciences) and CaptoMMC (HE Healthcare), have been evaluated for their ability to capture rhApoA1.

3.3.2.1. PURIFICATION OF rhApoA1 BY HEA HYPERCEL

The ligand HEA HyperCel (Hexylamine) has been well documented for the capture of several proteins (Ranjini et al. 2010; Pezzini et al. 2014). It is hypothesised that the nature of interaction between proteins and the HEA HyperCel ligand is a combination of hydrophobic and electrostatic. Equilibration is typically under certain amount of conductivity to promote hydrophobic attraction (Fig 3.5). A combination of salt and pH is crucial for reducing this hydrophobic attraction and promoting electrostatic repulsion to elute the bound protein (Pezzini et al. 2015).

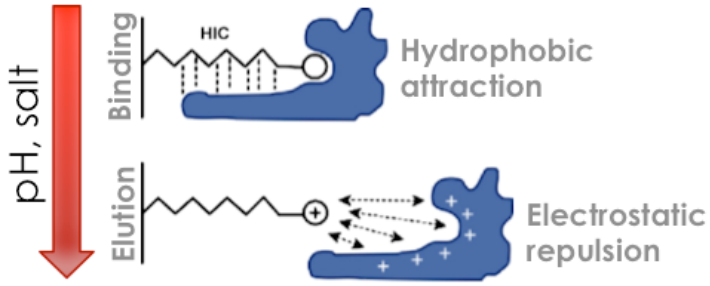


Figure 3.5. Hypothetical mechanism of interaction of target protein with HEA HyperCel ligand. Binding is promoted by hydrophobic attraction under relatively higher pH and salt concentrations. Subsequently, the bound proteins are eluted by lowering the pH and salt concentrations which in turn promotes electrostatic repulsion.

In this study, a certain amount of salt (250 mM NaCl) was added to the equilibration buffer to generate the same conductivity as that of the *P. pastoris* expression broth, in an attempt to reduce the number of sample processing steps prior to injection. The pH of the *P. pastoris* broth containing rhApoA1 was raised to 7.4 and subsequently injected on to the column. After passing the sample, the non-retained proteins were allowed to flow through, after which the salt in the buffer was eliminated in a linear gradient. Finally, elution was achieved by lowering the pH sequentially (Fig 3.6(a)).

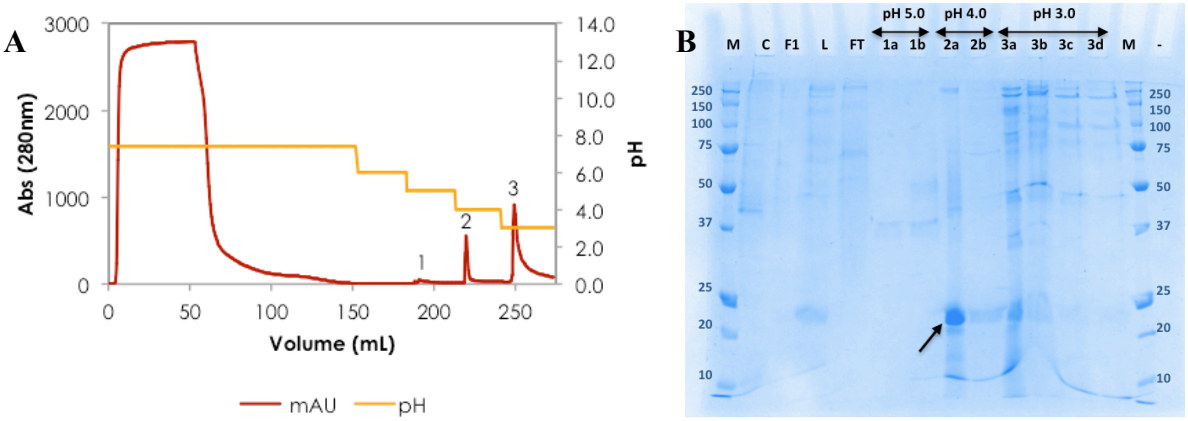


Figure 3.6. Purification of rhApoA1 by HEA HyperCel. (A) Chromatogram and (B) 12% SDS-PAGE analysis of peak fractions.

Upon 12% SDS-PAGE analysis, it was evident that the bound rhApoA1 was recovered at pH 4.0 (fraction 2a, indicated by the arrow). Some of the higher molecular weight proteins were loosely bound and were eluted at pH 5.0, whereas other proteins were more strongly bound to the ligand and eluted out at pH 3.0. The method was efficient in recovering rhApoA1 with an overall yield of 56.25% (Table 3.2), and was reproducible.

However, eluting rhApoA1 at pH 4.0 was a cause of concern. Recent studies have reported the propensity of ApoA1 to form amyloid like structures at pHs 4.0 and below (Ramella et al. 2012). It was hence imperative to minimise the exposure of rhApoA1 to very acidic pHs.

3.3.2.2. PURIFICATION OF rhApoA1 by PPA HYPERCEL

The PPA HyperCel ligand (Pall Life Sciences) is a phenyl propyl amine derivative (Table 3.1), and has been demonstrated in the efficient capture of a number of proteins over a wide pH range (Ranjini et al. 2010).

In this study, the binding and elution conditions tested for purifying rhApoA1 was similar to that with HEA HyperCel. The pH of the same was adjusted to 7.4 and loaded on to an equilibrated PPA HyperCel column. After binding, the salt was eliminated through a linear gradient and bound proteins were subsequently eluted in a step-wise manner by lowering the pH, yielding the chromatogram in Fig. 3.7(a).

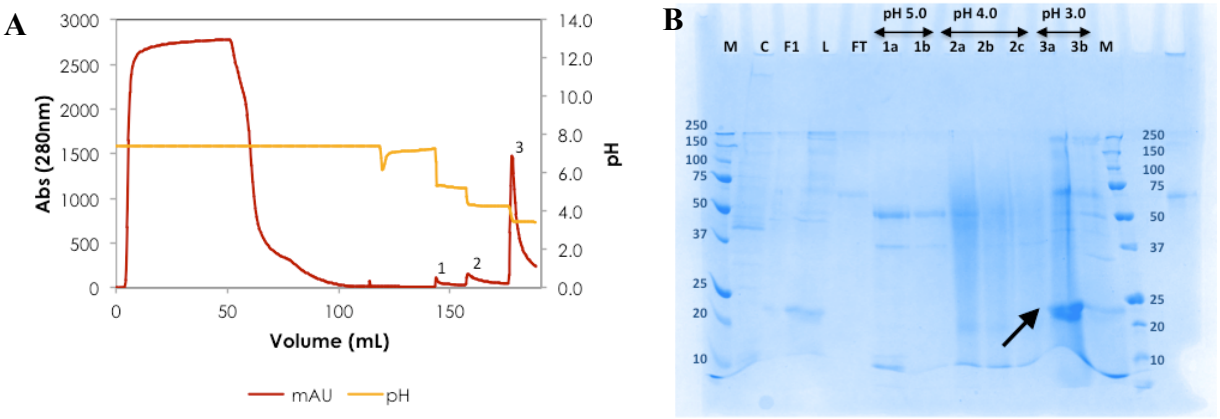


Figure 3.7. Purification of rhApoA1 by PPA HyperCel. (A) Chromatogram and (B) 12% SDS-PAGE analysis of peak fractions.

In case of PPA HyperCel, the bound rhApoA1 was eluted only at pH 3.0, which indicated much stronger binding than previously observed with HEA HyperCel. A few of the strongly bound proteins also co-eluted with rhApoA1 upon lowering the pH (lane 3a, Fig. 3.7(b)).

A quick comparison the chromatograms obtained with HEA and PPA HyperCel resins (Fig. 3.6(a) and Fig. 3.7(a)) would reveal differences in binding performances. Under the same binding conditions, PPA HyperCel absorbed rhApoA1 with much greater affinity owing to its greater hydrophobicity. ApoA1 being a predominantly hydrophobic protein (it is a lipid-binding protein) exhibits greater affinity to the phenyl-derivative (PPA) over the alkyl-derivative (HEA). Such comparisons have been extensively carried out in the past with other proteins (Ranjini et al. 2010; Pezzini et al. 2014; Pezzini et al. 2015), and the present data is consistent with literature.

In this work, HEA and PPA HyperCel ligands have both been efficient in selectively capturing rhApoA1 from the *P. pastoris* expression broth, but have demonstrated other difficulties in handling of the purified proteins, namely the lowering of pH to elute the bound protein. One possible solution is to rapidly titrate the collected elution fractions with a concentrated stock of Tris buffer at pH 8.5 to raise the pH to near-neutral levels.

3.3.2.3. PURIFICATION OF rhApoA1 BY CAPTO MMC

The multimodal Capto™ MMC ligand supports binding of proteins through hydrophobic, ionic and thiophilic interactions. Being a weak cation exchanger, it was expected to function in an inverse manner to the earlier tested HEA Hypercel (Section 3.3.2.1.). The column was equilibrated as described in the experimental section (acetate buffer at pH 5.0 containing 250 mM NaCl), which was the same pH and conductivity as that of the *P. pastoris* expression broth. Thus no sample pre-treatment was required prior to injection of the sample on to the column. This is especially an added advantage when designing a process of industrial relevance.

Following sample injection, the absorbance at 280nm was monitored until the signal reached baseline. The elimination of salt over a linear gradient didn't cause any proteins to unbind, in line with the expected salt-independent binding behaviour of Capto™ MMC. Subsequently, clear sharp peaks were observed when the pH was raised to 7.0 and 8.5 in steps (Fig. 3.8(a)). Any strongly bound proteins were then washed out with 0.5M NaOH (CIP).

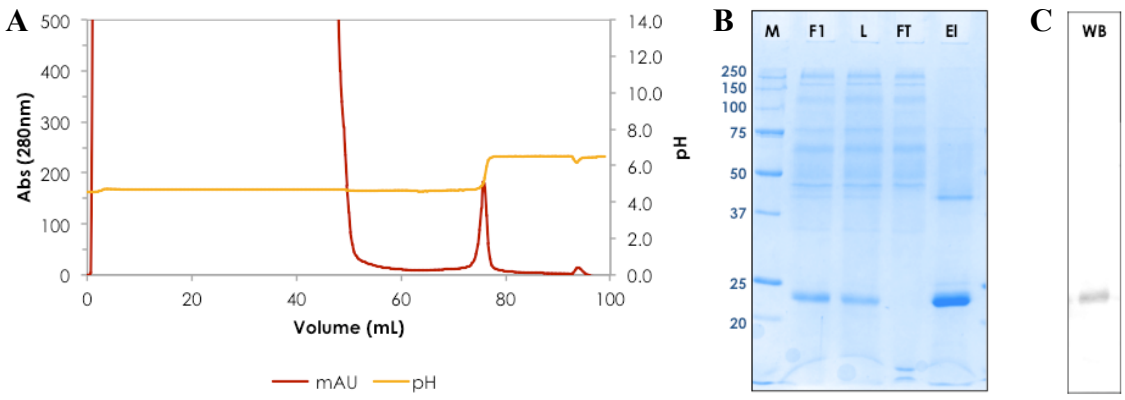


Figure 3.8. Purification of rhApoA1 by Capto MMC. (A) Chromatogram, (B) 12% SDS-PAGE analysis of peak fractions and (C) Western blot analysis of eluted fraction at pH 7.0.

All collected fractions were analysed over 12% SDS-PAGE (Fig. 3.8(b)), and clearly revealed complete adsorption of rhApoA1 under the given binding conditions. The protein was still bound after elimination of salt in the buffer, and eluted by raising the pH to 7.0 (lane E). In addition to the predominant ApoA1 band in the 25 kDa range, there was an additional band at roughly twice the

m.wt. Although this band did not provide a signal with anti-ApoA1 antibodies in western blots (possibly due to lower quantity), they were confirmed to be rhApoA1-dimers by mass spectrometric analyses (in-gel digestion; data not shown). To sum up, rhApoA1 was successfully purified to 84% purity using Capto™ MMC in a single step with 69% yield. The purification efficiencies of both purification methods have been summarized in Table 3.2.

Binding at mildly acidic pH (lower than the pI of rhApoA1) is consistent with earlier literature on the Capto™ MMC ligand. Being a salt-tolerant ligand, Capto™ MMC was able to retain the bound protein despite the elimination of conductivity. Subsequently, upon a rise in pH (resulting in a reversal of charges on the surface of the target protein rhApoA1), the protein desorbed and eluted out in a single sharp peak. This result was highly reproducible over several runs and was subsequently scaled-up to expanded bed mode (Chapter 4).

3.3.3. COMPARISON OF NOVEL PURIFICATION METHODS WITH PREVIOUSLY PUBLISHED METHODS

While previously published methods discussed in this chapter (Section 3.3.1) show reasonable efficiency in recovering rhApoA1 from *P. pastoris* expression broth, their efficiency in recovering maximal rhApoA1 in minimal number of steps is not suitable for further scaling up. The use of Triton X-114 to extract the hydrophobic rhApoA1 poses serious difficulties in eliminating all traces of the detergent without compromising on protein activity; precipitation with cold acetone is not pragmatically feasible for industrial scale-up, especially with large quantities of acetone and long incubation times that are required; the improvised ion-exchange chromatography method required extensive desalting prior to treatment, which is again not practically scalable.

The defined mixed-mode ligands that have been tested in this work, show great promise in capturing rhApoA1 directly out of the *P. pastoris* expression broth with minimum number of processing steps. The relative efficiencies of all the methods that have been compared are summarised below in Table 3.2.

Table 3.2. Comparison of purification efficiencies of the various tested methods

S. No.	Purification Method Employed	Number of Steps	Yield	rhApoA1 purity*	Reference
1.	Cloud-point extraction with Triton X-114	2	55.97%	57.1%	(Marco Aurélio Zezzi Arruda et al. 2011)
2.	Cold-acetone precipitation	14	60.00%	71.9%	(Feng et al. 2006)
3.	Ion-exchange chromatography	3	32.45%	42.6%	*Present work*
4.	Mixed-mode chromatogr. (HEA HyperCel)	1	56.25%	70.2%	*Present work*
5.	Mixed-mode chromatogr. (PPA HyperCel)	1	52.50%	76.3%	*Present work*
6.	Mixed-mode chromatogr. (CaptoMMC)	1	68.89%	84.0%	*Present work*

*Purity of the recovered rhApoA1 described based on densitometric analysis of the elution fraction on 12% polyacrylamide gel.

From the comparative data above, it was evident that the mixed-mode ligands were more effective in recovering rhApoA1, with Capto MMC (row # 6) proving to be most efficient in capturing rhApoA1 in a single step.

3.3.4. MASS SPECTROMETRIC ANALYSIS OF PURIFIED rhApoA1

The elution fractions from the HEA HyperCel, PPA HyperCel and CaptoMMC chromatographic experiment were subjected to in-solution digestion as described in the methods section, followed by analysis in an Agilent G6540A MS/MS Q-TOF mass spectrometer. The eluted fractions were independently subjected to trypsinisation and MS/MS analysis. Upon analysing the results using Agilent MassHunter software, each of the analysed fractions showed at least 65% sequence coverage with that of rhApoA1, confirming that the purified protein was indeed rhApoA1. The sequence coverage and list of identified peptides with their scores are detailed in figure 3.9 and table 3.3 respectively.

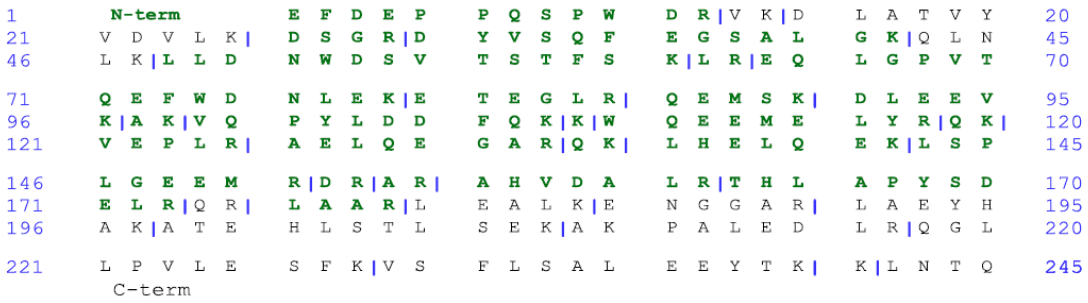


Figure 3.9. Sequence coverage of rhApoA1 identified by trypsinisation followed by ESI-MS/MS

Table 3.3. Peptides identified by ESI-MS/MS analysis of trypsinised rhApoA1

RT	Mass	Volume	Sequence	Seq Loc	Tgt mass	Δppm
7.803	1501.6410	840101	EFDEPPQSPWDR	A(1-12)	1501.6474	-4.27
7.032	1426.6506	112539	KWQEEMELYR	A(109-118)	1426.6551	-3.15
7.580	1410.6537	746003	KWQEEMELYR	A(109-118)	1410.6602	-4.60
7.421	1298.5511	105151	WQEEMELYR	A(110-118)	1298.5601	-6.99
7.933	1282.5579	523138	WQEEMELYR	A(110-118)	1282.5652	-5.72
6.139	868.5083	358149	QKVEPLR	A(119-125)	868.5131	-5.44
6.830	1722.9331	3165594	QKVEPLRAELQEGAR	A(119-133)	1722.9377	-2.65
6.439	612.3543	85412	VEPLR	A(121-125)	612.3595	-8.45
7.100	1466.7790	1286701	VEPLRAELQEGAR	A(121-133)	1466.7842	-3.52
6.045	872.4314	629248	AELQEGAR	A(126-133)	872.4352	-4.35
5.873	1151.6273	2036858	QKLHELQEK	A(134-142)	1151.6299	-2.25
6.008	1151.6228	33092	QKLHELQEK	A(134-142)	1151.6299	-6.12
6.021	895.4723	511582	LHELQEK	A(136-142)	895.4763	-4.53
6.157	895.4713	18084	LHELQEK	A(136-142)	895.4763	-5.60
7.025	1046.5024	141347	LSPLGEEMR	A(143-151)	1046.5067	-4.06
6.729	1317.6277	294618	LSPLGEEMRDR	A(143-153)	1317.6347	-5.29
5.940	1007.5575	50517	ARAHVDALR	A(154-162)	1007.5625	-4.91
6.140	780.4229	1904486	AHVDALR	A(156-162)	780.4242	-1.71
7.059	1300.6379	2800555	THLAPYSDELR	A(163-173)	1300.6412	-2.50
5.399	429.2688	720624	LAAR	A(176-179)	429.2700	-2.67
7.761	1814.8367	1041027	DSGRDYVSQFEGSALGK	A(26-42)	1814.8435	-3.77
8.044	1399.6581	1325726	DYVSQFEGSALGK	A(30-42)	1399.6620	-2.73
8.501	1611.7716	1078052	LLDNWDSVTSTFSK	A(48-61)	1611.7781	-4.02
8.568	2201.1047	1745984	LREQLGPVTQEFWDNLEK	A(62-79)	2201.1117	-3.18
8.940	2886.4404	830148	LREQLGPVTQEFWDNLEKETEGLR	A(62-85)	2886.4512	-3.74
8.813	1931.9205	1018736	EQLGPVTQEFWDNLEK	A(64-79)	1931.9265	-3.13
9.166	2617.2561	743511	EQLGPVTQEFWDNLEKETEGLR	A(64-85)	2617.2660	-3.80
9.283	3220.5189	870910	EQLGPVTQEFWDNLEKETEGLR QEMSK	A(64-90)	3220.5347	-4.89
6.076	703.3473	374034	ETEGLR	A(80-85)	703.3501	-3.90
6.444	1306.6131	606019	ETEGLRQEMSK	A(80-90)	1306.6187	-4.32
7.436	2019.9696	620866	ETEGLRQEMSKDLEEVK	A(80-96)	2019.9783	-4.28
3.007	621.2742	95057	QEMSK	A(86-90)	621.2792	-8.14
6.711	1334.6305	217153	QEMSKDLEEVK	A(86-96)	1334.6388	-6.18
6.617	731.3692	1813372	DLEEVK	A(91-96)	731.3701	-1.33

6.283	930.4967	36583	DLEEVKAK	A(91-98)	930.5022	-5.90
7.386	1450.7386	181580	AKVQPYLDDFQK	A(97-108)	1450.7456	-4.86
7.722	1251.6099	1769877	VQPYLDDFQK	A(99-108)	1251.6136	-2.89
7.346	1379.7035	1334726	VQPYLDDFQKK	A(99-109)	1379.7085	-3.68

Although extensive sequence coverage was observed in the MS analysis (greater than 65%), there were no tryptic peptide matches in the last 60 amino acids at the C-terminal, which hinted towards a possible truncation in the expressed protein.

Furthermore, MALDI-TOF analysis (Fig. 3.10) of intact undigested purified rhApoA1 revealed a lower molecular mass observed (21.99 kDa) than the expected molecular mass of 28.3 kDa.

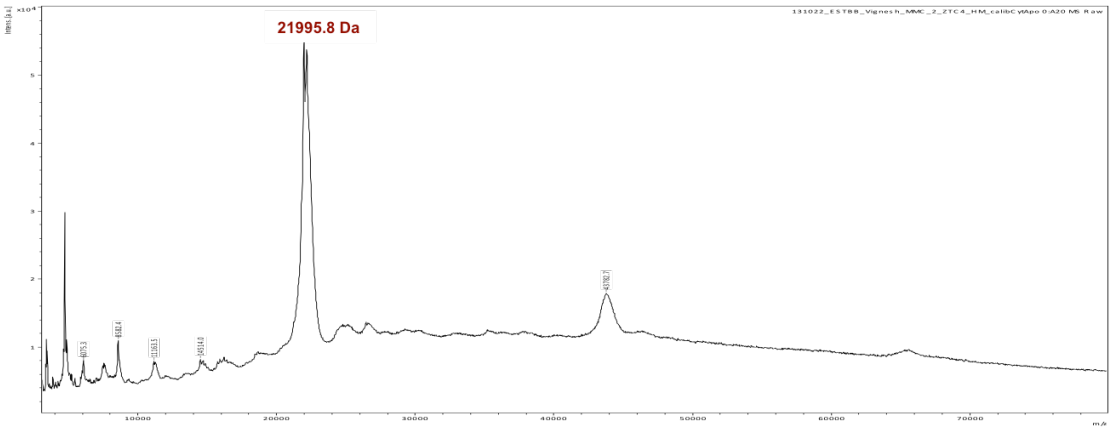


Figure 3.10. MALDI-TOF spectrum of intact purified rhApoA1 from *P. pastoris* X-33

Suspecting proteolytic activity by *P. pastoris*, the expression of the protein was also subsequently studied on a protease-deficient strain of *P. pastoris* (strain SMD1168) to compare the completeness of the proteins expressed by the two strains (Chapter 4).

3.4. CONCLUSION

In this chapter, a number of novel purification strategies have been elucidated and experimented to recover rhApoA1 from *P. pastoris* expression broth, and have been compared with methods that have previously been published in

literature. The comparative data clearly highlight the benefits and efficiencies of the mixed-mode chromatography based novel purification methods in capturing rhApoA1 from the *P. pastoris* expression broth, and their industrial applicability. Among the three resins supported, Capto MMC showed most promise and ease of application in the single-step recovery of rhApoA1, with zero pretreatment of *Pichia pastoris* expression broth. Subsequently, this purification method has been scaled-up (detailed in Chapter 4), and enabled the development of an integrated process.

Mass spectrometric identification of the purified rhApoA1 molecule revealed a possible truncation in the C-terminal region of the protein. It was hypothesised that the truncation (if existent) would have been due to proteolytic activity of proteases secreted by *P. pastoris* into the medium. In order to verify this hypothesis, an additional comparative study was carried out, by expressing the rhApoA1 protein in a protease-deficient strain of *P. pastoris*: SMD 1168. This comparative study has been further discussed in Chapter 4 of this thesis.

Chapter 4

Cloning, Expression & Purification of rhApoA1 in *P. pastoris* protease deficient strain SMD-1168

4.1. INTRODUCTION TO STRAINS OF *P. pastoris*

Pichia pastoris is a versatile microorganism that is being widely exploited for the overexpression of recombinant proteins. Different proteins demand different phenotypes and features in the host. Common *P. pastoris* strains used in the heterologous expression of proteins are listed below in Table 4.1.

Table 4.1. Commonly employed strains of *P. pastoris* with phenotypes and applications

S.No.	Strain	Genotype	Application
1.	X-33	Wild type	Selection of Zeocin™-resistant expression vectors
2.	GS115	*his4*	Selection of vectors containing *HIS4*
3.	KM71	*his4, aox1::ARG4, arg4*	Selection of expression vectors containing *HIS4* to generate Mut^s phenotype
4.	KM71H	*aox1::ARG4, arg4*	Selection of Zeocin™-resistant expression vectors to generate Mut^s phenotype
5.	SMD1168	*his4, pep4*	Selection of expression vectors containing *HIS4* to generate strains without protease A activity
6.	SMD1168H	*pep4*	Selection of Zeocin™-resistant expression vectors to generate strains without protease A activity
7.	$SuperMan_5$	$och1^-$, HIS^+	GlycoSwitch® strain, Man_5 N-linked oligosaccharide structures
8.	$SuperMan_5$	$och1^-$, his^-	GlycoSwitch® strain, Man_5 N-linked oligosaccharide structures (histidine auxotroph)

X-33 is the primary wild type strain, which is most widely used for applications that don't involve any glycosylation. The GS115 strain is obtained by knocking out *his4* gene, which enables a second level of selection on these strains in addition to the antibiotic resistance. KM71 strains generate Mut^s phenotypes upon transformation with an AOX1-promoter containing vector, which is suitable for high-density fermentations for the production of recombinant proteins that aren't sensitive to proteolytic degradation. On the other hand, the SMD1168 strains are protease A knockouts, which enable the expression of proteins that are sensitive to proteolytic degradation. In addition, there are new strains based on the GlycoSwitch® technology, which enable the expression of proteins with humanised-glycosylation patterns (Jacobs et al. 2008).

4.1.1. PROTEASE DEFICIENT STRAINS OF *P. pastoris*

Certain foreign proteins are unstable in the medium upon secretion by *P. pastoris*, primarily due to protease activity. In most cases, this degradation is attributed to major vacuolar proteases, which is more prominent in bioreactor cultures (J. M. Cregg et al. 2000). Protease deficient strains, such as SMD1168, have been successfully employed in the production of protease-sensitive proteins (Cereghino and Cregg 2000). In this chapter, the protease deficient *P. pastoris* strain SMD-1168 has been tested to confirm the possible degradation of rhApoA1 that has been discussed in Chapter 3.

4.2. EXPERIMENTAL

4.2.1. MATERIALS

Protease deficient strain of *P. pastoris* (SMD-1168) was a kind gift from Dr. Saroj Mishra, Professor, Department of Biochemical Engineering & Biotechnology, Indian Institute of Technology Delhi.

The transformation of ApoA1 gene into competent SMD-1168 cells was carried out using an Eppendorf Multiporator (Eppendorf AG, Germany), under the yeast module. All buffers and reagents were made from analytical grade reagents either from Sigma-Aldrich (Bangalore, India), Sisco Research Limited (Mumbai, India) or HiMedia (Mumbai, India).

4.2.2. TRANSFORMATION OF ApoA1 GENE INTO COMPETENT *P. pastoris* SMD-1168

Preparation of competent SMD-1168 cells: A single colony of SMD-1168 was inoculated in 10 ml of YPD, and incubated overnight at 30ºC, 200 rpm. Subsequently, the culture was added to 90ml of fresh YPD and incubated at 30ºC, 200 rpm for 4-5 hrs until the OD_{600} reached 0.8 to 1.2. The cultures were centrifuged at room temperature, 5000 rpm, 10 mins, and resuspended in 20 ml of YPD. To this culture, 400 µl of filter sterilised 1 M HEPES, pH 8.0 was added (final concentration 20 mM) and 500 µl of 1 M DTT was added, and mixed well by shaking at 30ºC, 50-70 rpm for 20 mins. 20 ml of ice-cold sterile water was

then added to the culture, and then centrifuged at 4ºC, 5000 rpm for 10 mins. The pellet was resulspended in 20 ml of ice-cold 1 M sorbitol, and centrifuged at 4ºC, 5000 rpm for 10 mins. The pellet was again resuspended in ~100-200 µl of Sorbitol yielding approximately 500-600 µl of competent SMD-1168 cells.

Transformation of ApoA1 gene into competent SMD-1168 cells: 100 µl of competent SMD-1168 cells were taken in a fresh 1.5 ml microcentrifuge tube, to which approximately 1 µg of *PmeI*-linearised pPICZα-ApoA1 plasmid was added and were incubated in ice for 10 mins. The contents were then transferred to a sterile pre-chilled 0.2 mm electroporation cuvette, and pulsed using a Multiporator (Eppendorf) in the yeast module at 1500 V with a time constant (τ) of 5 ms. After the electroporation, 900 µl of ice-cold 1 M Sorbitol was added to the culture, and 200 µl was spread on YPD agar plates containing 100 µg/ml Zeocin.

Screening of positive transformants: All colonies growing on the transformed plate were replica-plated on increasing concentrations of Zeocin up to 2 mg/ml. The best growing clones were then selected for expression studies.

Verification of ApoA1 gene in the yeast genomic DNA: The integration of the ApoA1 gene into the *P. pastoris* genome was verified by colony PCR analysis as described in Section 2.2.4. After the colony PCR run, the samples were subjected to 0.8% agarose gel electrophoresis and subsequently stained with Ethidium Bromide.

4.2.3. EXPRESSION OF rhApoA1 IN *P. pastoris* SMD-1168

The expression of rhApoA1 in SMD-1168 was carried out by a modified Mut^s expression protocol. Briefly, a single colony was inoculated in 10ml of BMGY and was grown overnight at 30ºC, 220 rpm until the OD_{600} reached 10-11, and inoculated into 100 ml of BMGY to reach a starting OD_{600} of ~0.3. This culture was grown for 2 days at 30ºC, 250 rpm for biomass to gain and then centrifuged at 4ºC, 5000 rpm for 5 mins. The pellet was then weighed (x gms) and then resuspended in equal volume (x ml) of BMMY medium. Induction was then

carried out with 1% methanol being replenished every 24 hrs supplemented with 0.1x YP. After the expression, the samples drawn at period intervals were analysed by 12% SDS-PAGE.

4.2.4. PURIFICATION OF rhApoA1 BY MIXED-MODE CHROMATOGRAPHY

Purification rhApoA1 expressed in SMD-1168 was carried out similar to that optimised with rhApoA1 expressed in X-33. The Capto MMC column was first equilibrated with 50 mM acetate buffer, pH 5.0, 250 mM NaCl (binding buffer). The pH of the *P. pastoris* expression broth at the end of induction was adjusted to pH 5.0 and injected on to the column (~10 ml of *P. pastoris* expression broth). The non-retained flow through fraction was collected, and once the absorbance reached baseline, the salt was eliminated over a linear gradient using 50 mM acetate buffer, pH 5.0 over 20 column volumes. Elution was then carried out in steps using 50 mM phosphate buffer at pH 7.0 and 50 mM Tris buffer at pH 8.5. The eluted fractions were analysed by SDS-PAGE and western blots to verify the successful purification of the target protein (rhApoA1).

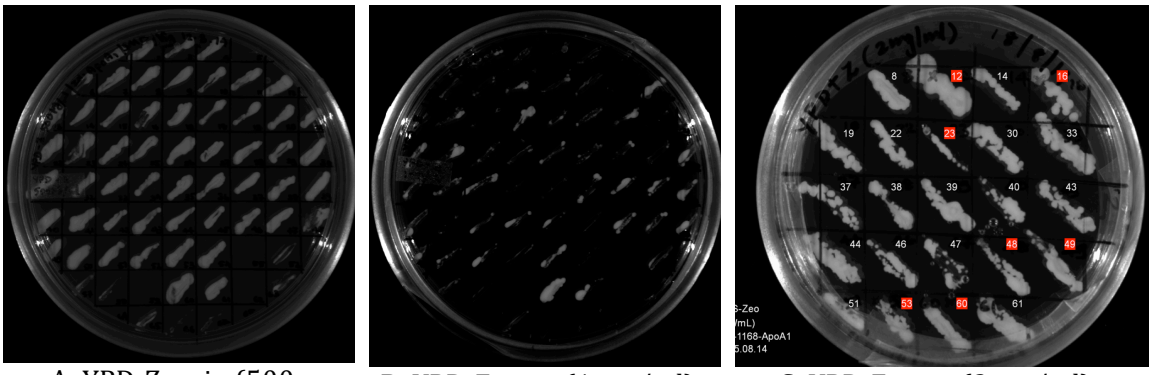
4.2.5. COMPARISON OF rhApoA1 EXPRESSED IN VARIOUS *P. pastoris* STRAINS

The intact masses of rhApoA1 expressed by the two different strains of *P. pastoris* (X-33 and SMD-1168) were compared by SDS-PAGE as well as mass spectrometry. Purified rhApoA1 fractions were measured by Agilent 6540 UHD Q-TOF mass spectrometer and analysed by Agilent Mass Hunter software to deconvolute and obtain intact mass of the measured fragments.

4.3. RESULTS

4.3.1. TRANSFORMATION OF pPICZα-ApoA1 AND SELECTION OF RESISTANT CLONES

The construct pPICZα-ApoA1 was linearised with *PmeI* and transformed into competent SMD-1168 strains as described in the methods section (Section 4.2.1). Subsequently, several colonies that exhibited resistance to Zeocin were screened with Zeocin concentrations of 500 µg/ml, 1 mg/ml and 2 mg/ml (Fig. 4.1).



A: YPD-Zeocin (500 µg/ml)

B: YPD-Zeocin (1 mg/ml)

C: YPD-Zeocin (2 mg/ml)

Figure 4.1. Replica plating of SMD-1168 cells transformed with ApoA1 gene at various concentrations of Zeocin

Five of the fastest growing clones exhibiting resistance to 2mg/ml (Fig. 4.1(c)) were further verified by colony PCR using ApoA1 gene-specific primers, as was done earlier (Section 2.3.1). Upon PCR and subsequent agarose gel electrophoresis (0.8% gel), all colonies tested positive for the integration of ApoA1 gene into the host genome (Fig. 4.2).

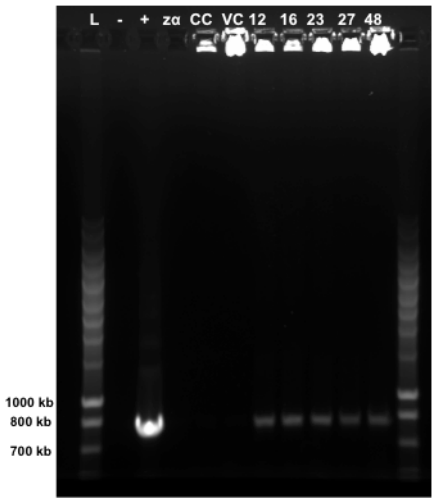


Figure 4.2. Colony-PCR analysis of cell control (CC), vector control (VC), and five SMD-1168-ApoA1 clones (#12, #16, #23, #27, #48).

After screening by colony PCR, one of the clones (#23) was taken further for expression studies (Section 4.3.2).

4.3.2. EXPRESSION OF rhApoA1 IN *P. pastoris* SMD-1168

Expression of rhApoA1 in SMD-1168 was carried out as described in methods section (Section 4.2.3). The OD_{600} of the cells were periodically checked for monitoring growth, and samples drawn every 24hrs were analysed over 12%

SDS-PAGE (Fig. 4.3) and stained with coomassie blue, which unequivocally demonstrated the successful expression of rhApoA1 in SMD-1168.

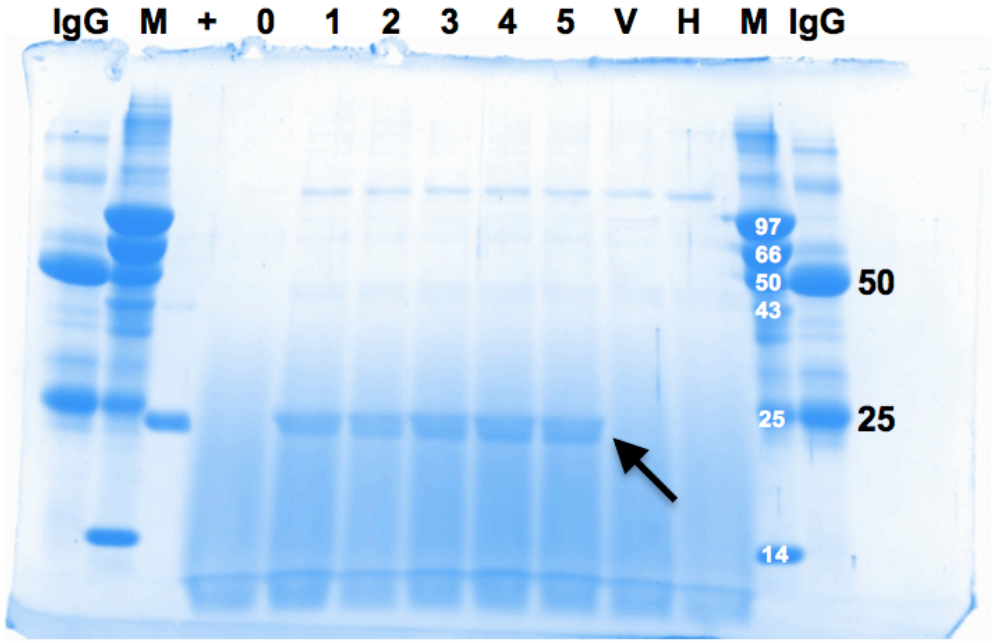


Figure 4.3. Expression of rhApoA1 on SMD1168: lanes correspond to Marker (M), reduced IgG as reference marker (IgG), ref. rhApoA1 produced in in X-33 (+), non-induced (0) & induced samples from 1-5 days, host transformed with just vector (V) and just host (H) induced for 5 days

As is evident from Fig. 4.3, there was a consistent increase in intensity of the band corresponding to rhApoA1 with increasing duration of induction. Furthermore, rhApoA1 expressed and purified from X-33 was run as a reference protein to check for any apparent differences in molecular weight. A priori, there didn't seem to be a significant electrophoretic difference in molecular weight between the rhApoA1 expressed in X-33 and SMD-1168. Nevertheless, in order to unequivocally confirm the difference in size of the expressed rhApoA1 molecule, they were also analysed by mass spectrometry (Section 4.3.4).

4.3.3. PURIFICATION OF rhApoA1 BY MIXED-MODE CHROMATOGRAPHY

Upon successful expression of rhApoA1, ~15 ml of the expression broth was subjected to mixed-mode chromatography using CaptoMMC, as described in the experimental section (Section 4.2.4).

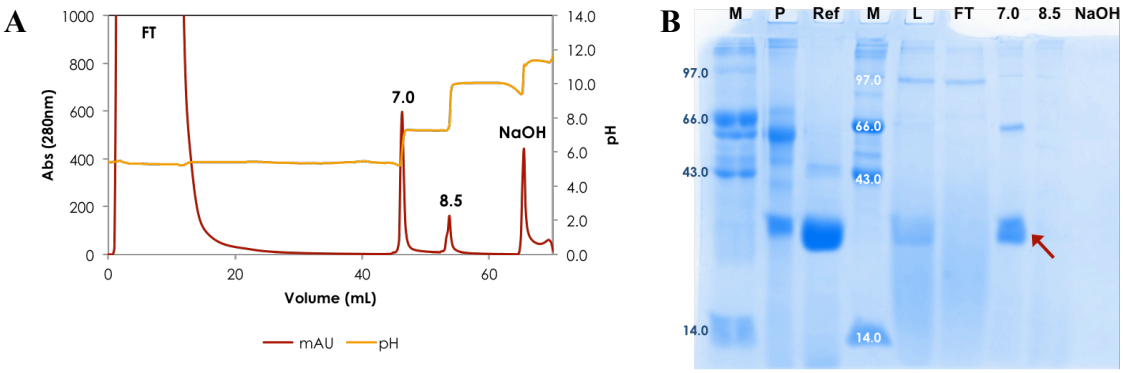


Figure 4.4. Purification of rhApoA1 expressed in *P. pastoris* SMD-1168 by CaptoMMC. (A) Chromatogram and (B) 12% SDS-PAGE analysis of load (L), non-retained (FT), and elution fractions at pHs 7.0 & 8.5. Plasma derived ApoA1 (P) and rhApoA1 expressed in *P. pastoris* X-33 (Ref) were added as controls for comparing m.wt.

The purification profile (Fig. 4.4(a)) was identical to that obtained earlier with rhApoA1 being purified from *P. pastoris* X-33. 12% SDS-PAGE analysis (Fig. 4.4(b)) again indicated towards no significant difference in molecular weight between rhApoA1 expressed in SMD-1168 and X-33 strains of *P. pastoris* and ApoA1 recovered from plasma (lane P).

4.3.4. COMPARISON OF rhApoA1 EXPRESSED IN X-33 AND SMD-1168

In order to unequivocally conclude on any potential truncation in rhApoA1 expressed in *P. pastoris*, the proteins expressed in different strains were compared by mass spectrometry.

Purified fractions containing rhApoA1 obtained from *P. pastoris* X-33 and SMD-1168 were analysed by 6540 UHD Q-TOF mass spectrometer (Agilent) and major species found in both samples were compared (Fig. 4.5). The major species in the purified rhApoA1 fraction from X-33 (22862.24 Da) was nearly identical to the major species in the purified rhApoA1 fraction from SMD-1168 (22869.30 Da). This clearly showcased that that was no significant difference in molecular weight between the rhApoA1 proteins expressed in *P. pastoris* strains X-33 and SMD-1168.

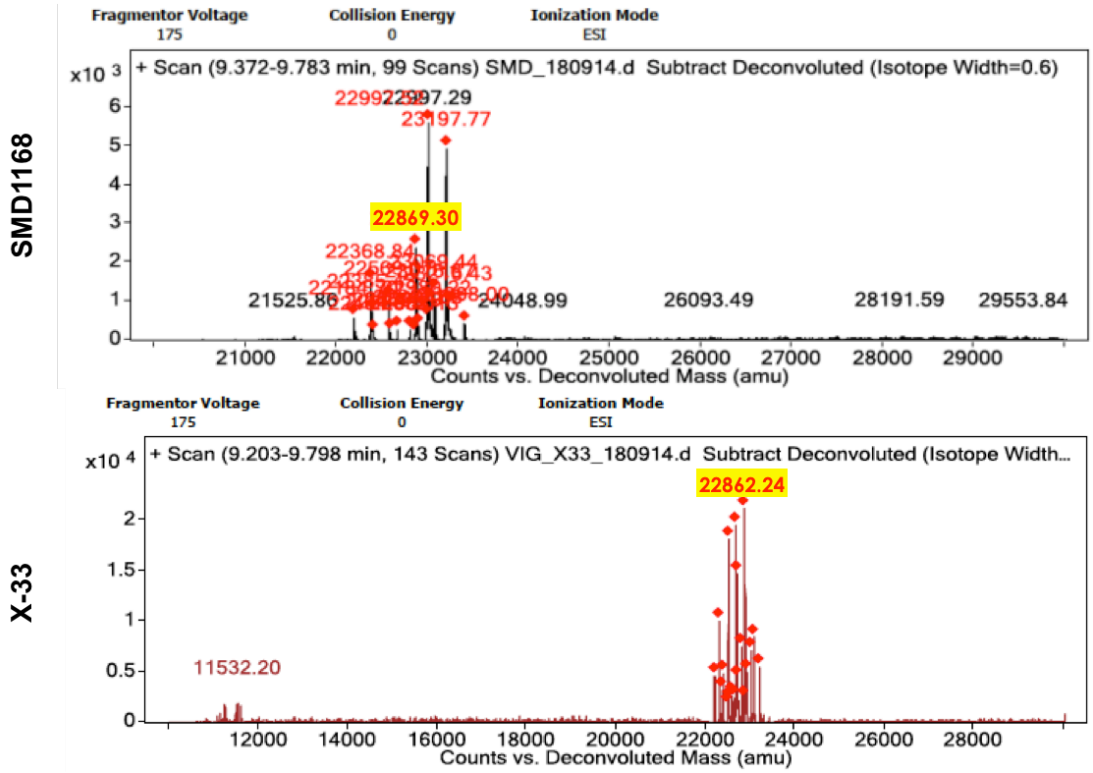


Figure 4.5. ESI-Q-TOF analysis of purified rhApoA1 fractions from *P. pastoris* X-33 and SMD-1168

Subsequently, the intact mass of rhApoA1 expressed in *P. pastoris* X-33 was measured by MALDI-TOF mass spectrometry (Fig 4.6(a)), and compared to ApoA1 recovered from plasma previously reported in literature (Feng et al. 2006).

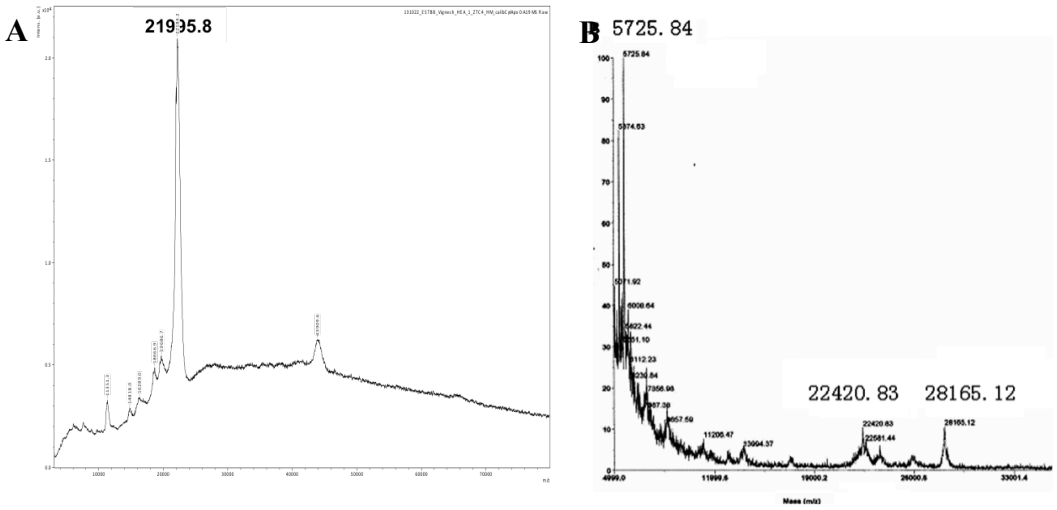


Figure 4.6. MALDI-TOF mass spectrometric analysis of (A) rhApoA1 expressed in *P. pastoris* X-33 and (B) plasma ApoA1 (Feng et al. 2006)

The MALDI-TOF profile of rhApoA1 from X-33 (Fig. 4.6(a)) showed a major species at 21995.8 Da, which was nearly identical to the major species reported earlier for plasma ApoA1 (22420.83 Da, Fig. 4.6(b)). Based on the above mass spectrometric comparisons, it was conclusive that there wasn't a significant difference in molecular mass between plasma and recombinant ApoA1 expressed in *P. pastoris* X-33 and SMD-1168 strains.

4.4. CONCLUSION

Truncation of recombinant proteins expressed in *P. pastoris* due to proteolytic activity has been reported earlier in literature (J. M. Cregg et al. 2000). In the present work, potential truncation was hypothesised owing to consistent lower molecular weight demonstrated by polyacrylamide gel electrophoresis (Chapter 3). Electrophoretic and mass spectrometric comparison of rhApoA1 expressed in X-33 with that expressed in SMD-1168 as well as plasma-derived ApoA1 clearly showcased no significance difference in molecular weight, indirectly confirming absence of any truncation in the expressed recombinant protein.

Chapter 5

Scale-up of production and purification of rhApoA1 using *P. pastoris* X-33

5.1. INTRODUCTION TO SCALE-UP OF INDUSTRIAL PROCESSES

Any bioprocess needs to be adequately scaled up to make it industrially viable. A typical bioprocess for the generation of recombinant therapeutics involves development of a stable strain expressing the target protein, followed by its production and subsequent purification. Scaling-up is typically carried out at both fermentation and downstream processing steps, with appropriate optimisation in order to maximise yield (Fig. 5.1).

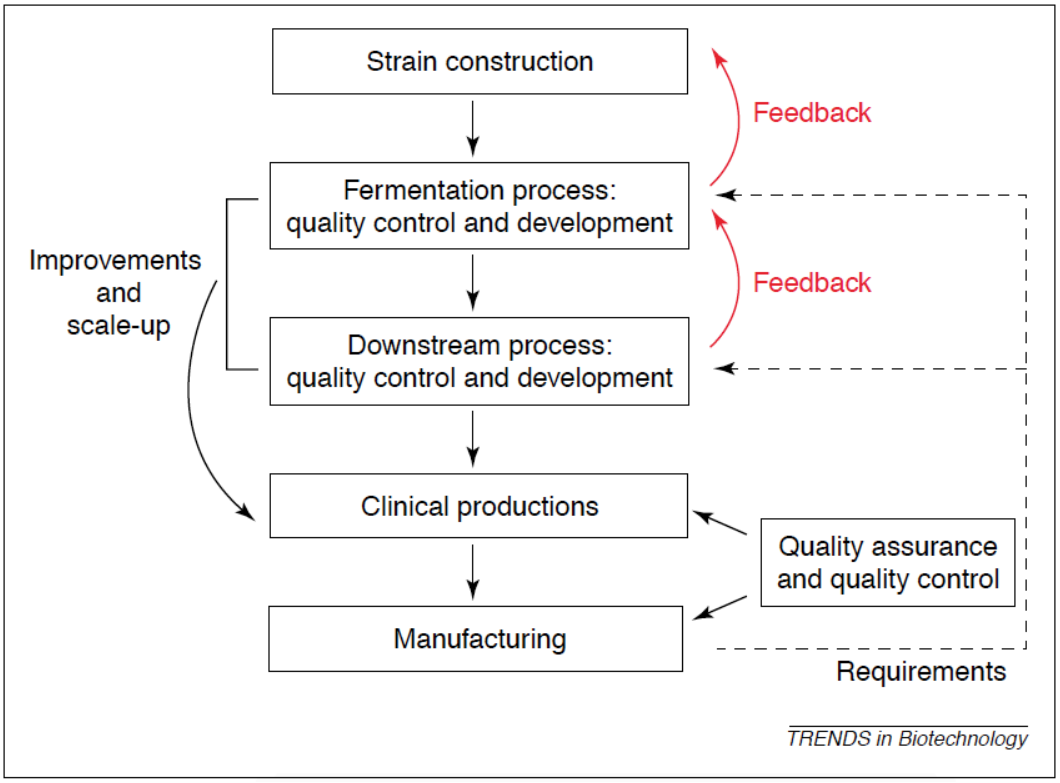


Figure 5.1. Development of biopharmaceutical product (Thiry and Cingolani 2002)

Various parameters need to be optimised during the process of scaling-up:

- Production/fermentation level: culturing conditions, feed rate, bioreactor size
- Purification level: sample pretreatment, chromatography characteristics

5.1.1. SCALE-UP OF EXPRESSION OF RECOMBINANT PROTEINS

A vast majority of industrial processes using genetically modified microorganisms to overexpress recombinant proteins use one of the three species: *Escherichia coli*, *Saccharomyces cerevisiae* and *Pichia pastoris* (Thiry and

Cingolani 2002). All three microorganisms showcase their relative advantages in terms of production levels, post-translational modifications, secretion capabilities, etc. In this work, the methylotrophic yeast *Pichia pastoris* has been exploited for the expression of rhApoA1.

During scale-up, the primary aim is in increasing the size and volume of expression, in addition to optimising culture conditions to maximise yield of the heterologous protein. Temperature, pH and dissolved oxygen are parameters that are typically optimised during expression and scale-up of production of recombinant proteins (Lerner-Marmarosh et al. 1999). A reduction in induction temperature and pH conditions helps enhance the amount of recombinant protein produced by reducing protease activity. In the present study, the temperature of induction and pH of the medium have been optimised to 28°C and 5.0, and the corresponding increase in yield have been discussed.

5.1.2. SCALE-UP OF PURIFICATION SYSTEMS

Industrial downstream processing of therapeutic proteins typically involves a clarification or pretreatment step followed by a preparative capture chromatography purification step and further polishing to derive the final purified protein, prior to filtration and formulation (Rathore and Velayudhan 2002). Important factors that dictate the efficiency of a scaled-up purification process are listed below (Garcia, Prazeres, and Cabral 2003):

- Quality of gel material: affinity for the target protein, physical & chemical stability, non-biodegradability, inertness to the product, incompressibility
- Quality of sample to be chromatographed (free of particles that could clog columns)
- Reduction/Elimination of protease activity that could affect target protein
- Foaming due to tensioactive agents

Sample pretreatment is especially important to ensure reproducibility of the process and reusability of the chromatographic resin. Alternate chromatographic approaches like fluidised beds or expanded beds could also be employed used for reducing the number of pretreatment, which in turn would reduce the

number of processing steps and in establishing a continuous process (Hjorth 1997).

5.1.3. EXPANDED-BED ADSORPTION FOR THE PURIFICATION OF PROTEINS

Expanded bed adsorption (EBA) allows clarification and capture of target protein in a single step. In an expanded bed, a particulate adsorbent in a column is allowed to rise from its settled state by applying an upward flow, and the sample is injected from below (Noubhani et al. 2002). Provided that the physical properties of the beads are significantly different from those of the particulates in the feedstock, the particulates can pass freely through the voids in the bed without becoming trapped (Mattiasson and Nandakumar 2000). This eliminates the need for pre-clarification of crude feedstock before application to packed beds using centrifugal or filtration techniques and also permits the recovery of particulate bioproducts using column purification techniques. Fig. 5.2 illustrates the underlying principle behind EBA.

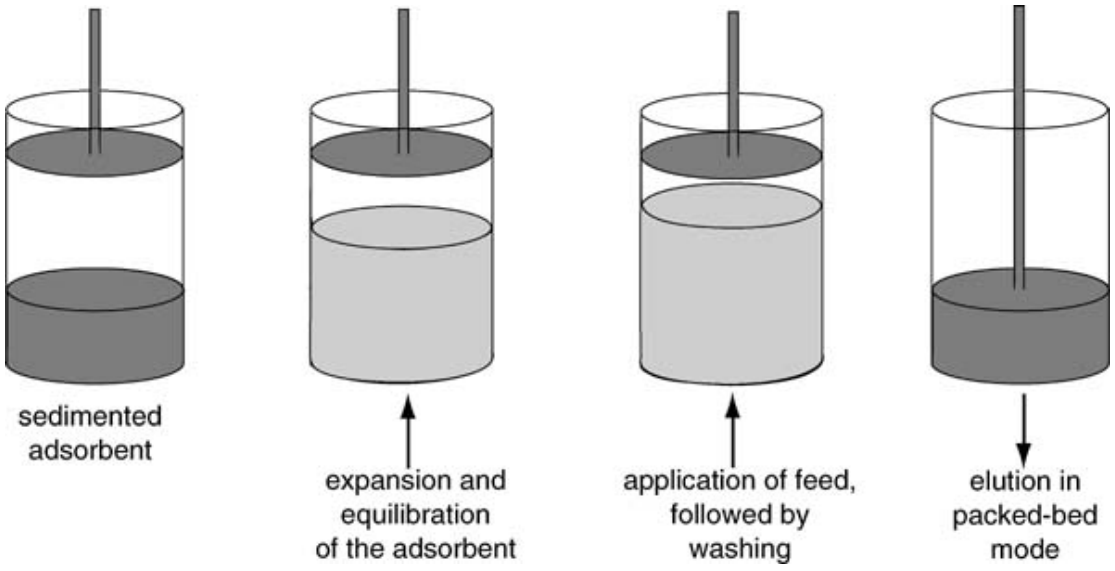


Figure 5.2. Resin bed status throughout the steps of an EBA chromatography experiment. Arrows indicate direction of feed flow. Cleaning in place (not shown) is the last step and is done in expanded-bed mode, similar to expansion and equilibration of the adsorbent. Reproduced from Amersham Biosciences Handbook.

The particle size and sedimentation velocity play an important role in tailoring the chromatographic characteristics of an adsorbent for use in expanded bed adsorption. EBA adsorbents like Streamline (GE Healthcare) are based on a

composite particle containing an inert core material that is surrounded by an organic shell, enabling them to have a high sedimentation velocity at a reasonable particle size. Typically, by altering the particle composition, a Gaussian like distribution of particle size and particle density is achieved in an expanded bed.

Fluidisation of particles occurs when particles are pushed upwards in a column at a velocity corresponding to their sedimentation velocity. The degree of expansion of the bed is controlled by a number of factors: the size and density of the adsorbent beads, the linear flow velocity of the mobile phase, and the viscosity of the mobile phase. The bed is usually expanded to 2-3 times the packed-bed height. The absolute value for the degree of expansion depends on liquid density and viscosity. An increase in the viscosity of the buffer system causes an increase in the degree of expansion (Mattiasson and Nandakumar 2000). Several recombinant proteins have been successfully purified by this method (Chase 1998; Hjorth 1997; Lamotte et al. 1999).

5.2. EXPERIMENTAL

5.2.1. MATERIALS

Scale-up expression studies in benchtop bioreactors were carried out on Sartorius-Stedim Biostat B-plus bioreactors (5l) monitored by MCSF Win data acquisition software (Sartorius). The Expanded Bed Adsorption experiments were carried out using Direct CST-1 resin (GE Healthcare, Uppsala, Sweden) loaded on a Streamline 25 housing (i.d. 2.5cm x 100cm, GE Healthcare, Uppsala, Sweden) controlled and monitored by Unicorn software (GE Healthcare, Uppsala, Sweden).

5.2.2. SCALE-UP OF PRODUCTION OF rhApoA1 TO 5L BENCH-TOP BIOREACTOR

After initial batches of bioreactor studies on 2l benchtop reactors, the clone F1 was further for scaled-up for production in a 5l benchtop bioreactor.

First, a single colony was inoculated and grown in baffled flasks containing 100ml of buffered glycerol-complex medium (BMGY, 100mM potassium phosphate buffer, pH 6.0, 13.4g/L YNB, $4x10^{-4}$g/L biotin, 10g/L glycerol and 150 µg/mL Zeocin™) up to an OD (600nm) of 4-8, and this was inoculated in 4L of BMGY in a BIOSTAT® Bplus laboratory 5l bioreactor with a starting OD_{600} of about 0.3. The temperature and pH were maintained at 30°C and 6.0 respectively, and dissolved oxygen level was maintained at 15% saturation by regulating aeration and agitation in a cascade manner. After complete consumption of glycerol in the medium (verified by standard glycerol assay; data not shown), a methanol fed-batch phase was initiated by adding methanol every 8hrs to a final concentration of 0.5%. Samples were drawn every 24hrs to follow the growth profile and protein production. After 120hrs of induction, the culture was harvested and directly chromatographed over an equilibrated Direct CST-1 column in Expanded Bed mode (Section 5.2.3). The expression of the recombinant protein was also monitored by SDS-PAGE and Western Blot analysis with samples drawn periodically (every 24hrs) from the bioreactor.

5.2.3. PURIFICATION OF rhApoA1 BY DIRECT CST-I IN EXPANDED-BED MODE

Once favourable conditions were achieved for the recovery of rhApoA1 using Capto™ MMC (Section 3.3.2.3), scale-up of purification was carried out in Expanded Bed mode using Direct CST-1 (GE Healthcare), which has the same chemistry as Capto™ MMC. The buffer conditions were as optimised in preliminary experiments. The column was equilibrated using 50mM acetate buffer, pH 5.0 and expanded by sequentially increasing flow-rate. The expression culture at the end of a fermentation batch (containing cells) was directly injected on to the expanded column to capture our target protein. Once binding and wash steps were completed, the column was allowed to settle and elution was carried out in downward flow using the same elution buffers. The various fractions were analysed by SDS-PAGE and western blots to verify the presence of rhApoA1.

As a polishing step, the elution fractions from the EBA chromatographic run were subjected to ion-exchange chromatography using Resource Q anion-exchange column (GE Healthcare). The column was equilibrated with 20mM

phosphate buffer, pH 7.0, after which the sample (fraction #2 from EBA experiment) was injected on to the column. After the absorbance at 280nm reached baseline, elution was carried out in steps containing 5%, 15% and 30% of 20mM phosphate buffer, pH 7.0, 1M NaCl. All collected fractions were analysed by 12% SDS-PAGE.

5.3. RESULTS

5.3.1. SCALED-UP EXPRESSION OF rhApoA1

Production of rhApoA1 in 5l benchtop bioreactor was carried out under conditions similar to that that were the maintained with the 2l benchtop bioreactor (Section 2.3.3). Briefly, an appropriate volume of preculture in BMGY containing 200µg/ml Zeocin was inoculated into a sterile bioreactor containing BMGY medium to a starting OD_{600} of 0.3. For the first 24hrs, cells were allowed to grow in the biomass accumulation phase in glycerol-containing medium (BMGY). Once all glycerol was exhausted, the pH of the medium was lowered to 5.0, and the induction phase was commenced with methanol being fed every 8hrs to a final concentration of 0.5% (Fig. 5.2, turquoise curve). This ensured consistent replenishment of methanol and continued production of the target protein (rhApoA1).

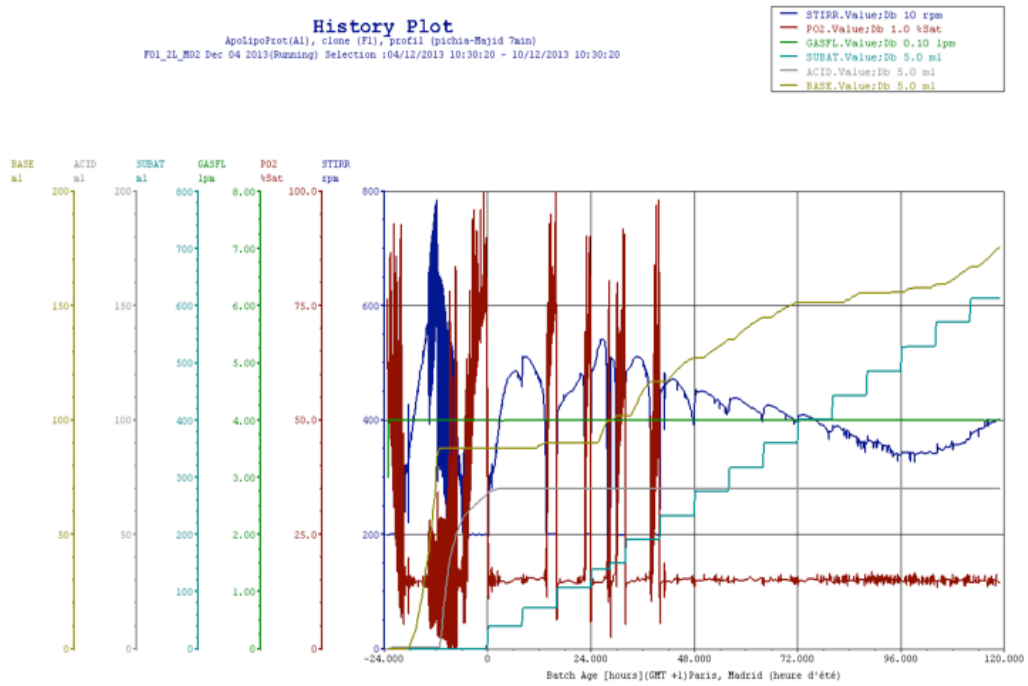


Figure 5.3. Expression profile of rhApoA1 production in 5l benchtop bioreactor.

As monitored with the 2l bioreactor production batch, the pH and temperature were maintained in real time, using sterile concentrated reservoirs of acid/base and heating jacket respectively. From Fig. 5.3, it is evident that the cells were consistently metabolising the substrate supplied, inferable from the curves corresponding to pO_2 levels (red) and agitation (blue). An increase in amount of base added to the bioreactor (yellow) is also an indication of secretion of proteins into the culture lowering the pH.

During the production, samples were drawn at various time points to check for glycerol consumption, target protein secretion, consistent growth and possible contamination. OD_{600} values of samples drawn at various time points revealed good biomass accumulation in the glycerol phase, and stable growth throughout the induction period (Fig. 5.4).

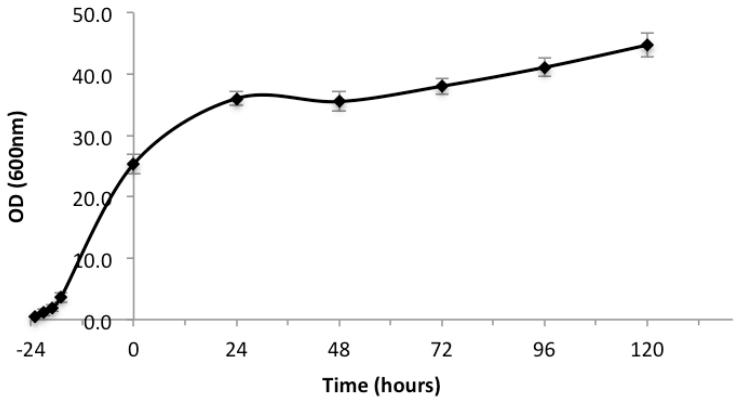


Figure 5.4. Optical Density (600nm) profile of samples drawn at various time points.

Furthermore, microscopic examination revealed stable budding of yeast cells up to the 5th day of production (Fig. 5.5) and the absence of any contamination. After the completion of the production batch, samples drawn at various time points were analysed by SDS-PAGE, which revealed consistent expression of rhApoA1 (Fig. 5.6), as observed earlier with the 2l bioreactor run.

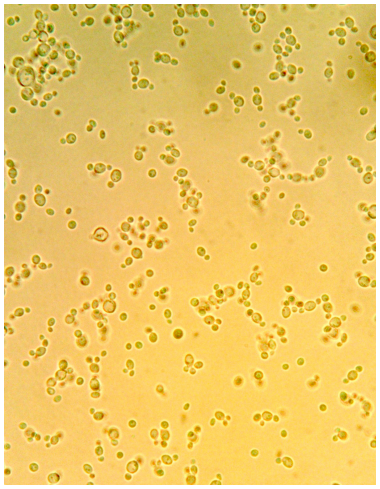


Figure 5.5. Microscopic examination of budding yeast cells (transformed *P. pastoris* X-33) at 40x magnification.

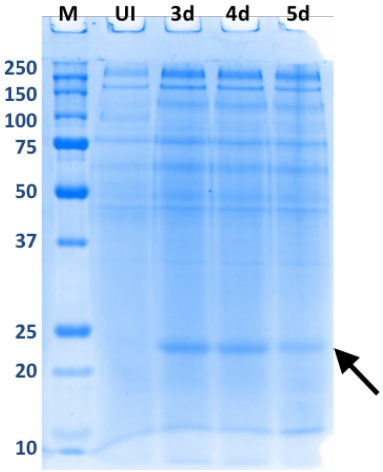


Figure 5.6. SDS-PAGE analysis of non-induced (UI) and samples drawn at 72h, 96h, and 120h of induction (3d, 4d and 5d, respectively). Band corresponding to rhApoA1 indicated by arrow.

The expression profiles of shake flask cultures were compared with bioreactor studies (Table 5.1), and the chemostat conditions clearly enabled the achievement of nearly twice the biomass (greater OD_{600}) and protein yield (43.8 mg/l of production).

Table 5.1. Comparison of production parameters at various scales of expression

Parameter\Scale	Flask	2l Bioreactor	5l Bioreactor
Volume of culture	150 ml	1800 ml	4000 ml
Harvesting OD_{600}	27.56	41.34	44.71
Induction pH	6.0	5.0	5.0
Buffering system	phosphate buffer	phosphate buffer + acid/base	phosphate buffer + acid/base
Induction temperature	30°C	28°C	28°C
rhApoA1 yield	22.4 mg/l	37.5 mg/l	43.8 mg/l

The entire culture from the 4l production batch was utilised for one batch of purification in Expanded Bed Adsorption (Section 5.3.2).

5.3.2. PURIFICATION OF rhApoA1 BY DIRECT CST-I

In order to test the industrial applicability of this purification method, an Expanded Bed Adsorption resin: Direct CST I, was tested for its efficiency in capturing rhApoA1 from a crude feed containing *P. pastoris* cells directly from the bioreactor. The Direct CST I ligand has the exact same chemistry of Capto™ MMC, and has earlier been characterised using BSA and Myoglobin as model proteins (Li et al. 2006).

Expanded bed adsorption enables the direct affinity capture of target proteins from crude samples. In our case, about 4 litres of unclarified *P. pastoris* culture (after a production batch in a benchtop bioreactor with 120hrs of induction, section 5.3.1) was injected to an expanded Direct CST I column. After the cell culture was completely passed through and the absorbance at 280nm reached baseline (washing completion), the flow was stopped and the column was allowed to settle. Subsequently, the salt was eliminated and pH was raised to elute bound proteins as earlier optimised in the packed column experiments with CaptoMMC (Section 3.3.2.3).

The chromatographic profile (Fig. 5.7a) was similar to that obtained with the packed 1ml HiTrap Capto™ MMC column (Fig. 3.8a). As expected, the peak eluted at pH 7.0 predominantly contained rhApoA1 (79% as quantified from the gel; Fig. 5.7b). There were still small amounts of other contaminating proteins (higher m.wt.), which needed to be eliminated.

An additional ion-exchange polishing step with Resource Q anionic exchanger column (GE Healthcare) was introduced. The polishing step was effective with 20mM sodium phosphate buffer (Fig. 5.7c), when rhApoA1 was eluted with just 50mM NaCl. At 300mM NaCl, other contaminating proteins co-eluted along with rhApoA1 and its dimer.

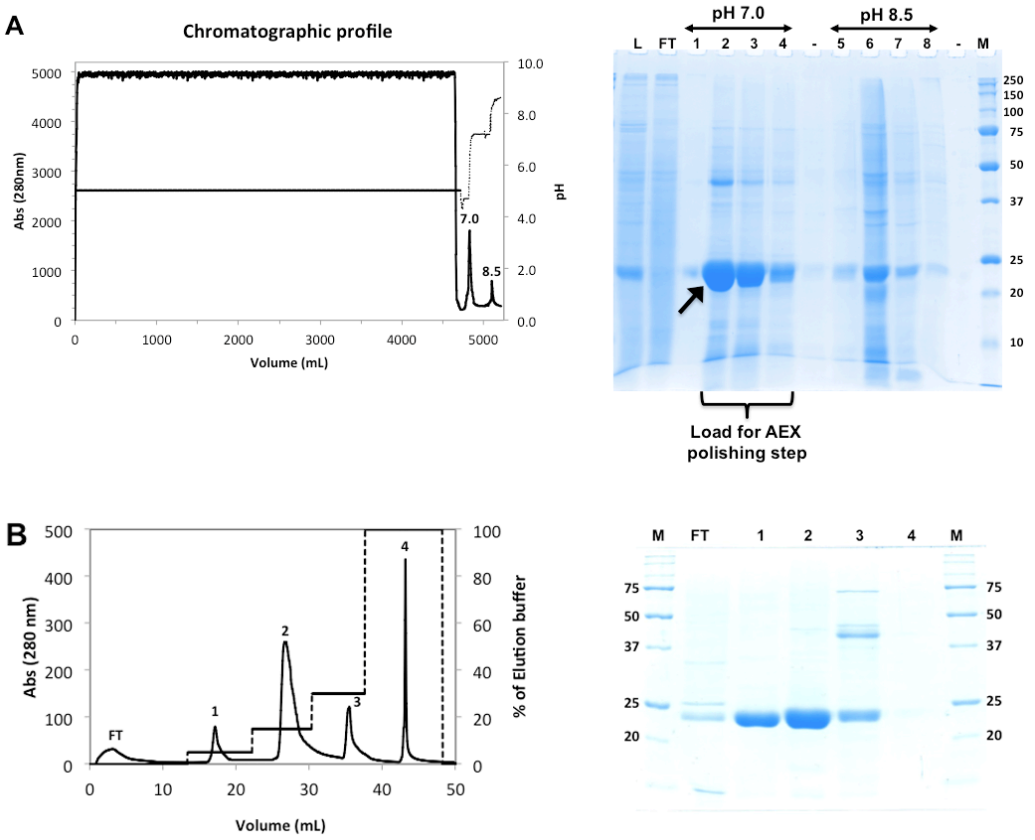


Figure 5.7. Purification of rhApoA1 by Direct CST-1 in Expanded Bed Adsorption mode. (A) Chromatogram and 12% SDS-PAGE of load (L), non-retained (FT) and eluted fractions at pHs 7.0 (fractions 1 – 4) and 8.5 (fractions 5 – 8). (B) Chromatogram and 12% SDS-PAGE analysis of fractions from Resource Q ion-exchange polishing step: load (L), non-retained (FT), and eluted fractions at 5%, 15%, 30% and 100% of elution buffer (20mM phosphate buffer, pH 7.0, 1M NaCl).

Table 5.2. Comparison of purification efficiencies of packed column & EBA modes

Scale	Purification method	Column size	rhApoA1 content (mg)		Yield (%)	Purity of rhApoA1*
			Load	Elution		
Packed column	Mixed-mode HEA HyperCel™	5ml prepacked column	2.24	1.26	56.25	70.2
Packed column	Mixed-mode Capto MMC™	1ml prepacked column	2.21	1.51	68.89	84.0
Scaled-up: EBA (2 steps)	Mixed-mode Direct CST I	118ml settled-bed volume	113.48	84.77	74.69	79.0
	Anion exchange Resource Q	1ml prepacked column				96.2

**Purity of the recovered rhApoA1 described based on densitometric analysis of SDS-PAGE.*

Overall, about 136 mg of rhApoA1 was successfully purified in a single Direct CST I Expanded Bed Adsorption experiment from approximately 4l of unclarified

expression culture to greater than 96% purity upon polishing with Resource Q. The purification efficiencies of packed-column and expanded-bed modes were compared (Table 5.2), and the scale-up was shown to be effective.

The scale-up of purification of rhApoA1 using Direct CST-I in expanded bed adsorption mode was unequivocally established, and this paved way to the development of an integrated process (Section 5.3.3) for the production and rapid one-step capture of rhApoA1 expressed in *P. pastoris*.

5.3.3. DEVELOPMENT OF AN INTEGRATED PROCESS FOR THE PRODUCTION & PURIFICATION OF rhApoA1

For any production/purification process to be industrially viable, the focus is always on minimising the number of processing steps while maximising recovery/yield. In this study, the rhApoA1 has been expressed without the addition of any affinity tag so that it may be generated close to its native conditions. The application of Expanded Bed Adsorption sorbent Direct CST-I enables the development of an integrated process (Fig. 5.8) to directly capture the expressed rhApoA1 protein straight out of the bioreactor without any pre-treatment or clarification.

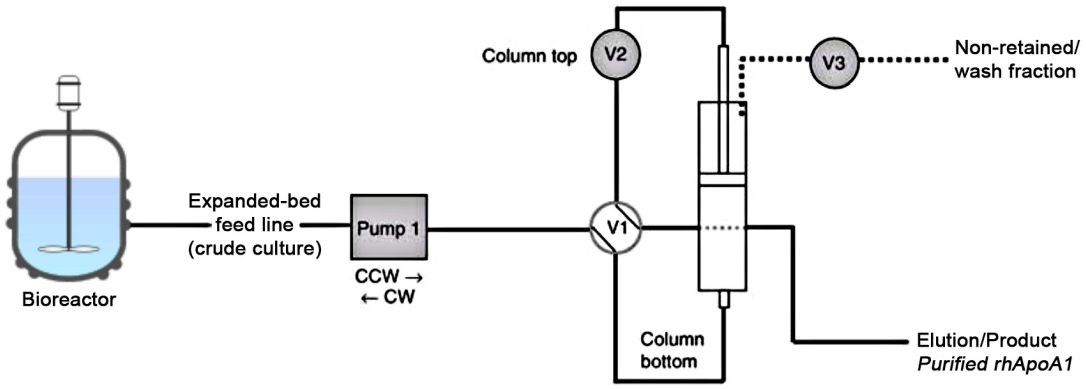


Figure 5.8. Integrated process for the direct purification of rhApoA1 from bioreactor using Direct CST-I in Expanded Bed Adsorption mode.

Briefly, the process involves the direct pumping of the crude culture feed from the bioreactor on to an expanded Direct CST-I column (Fig. 5.8). During binding, valve V1 is in upward flow position (as indicated in the figure), valve V2 is closed

and the non-retained and wash fractions flow through out by valve V3. Subsequently, after all binding and wash is complete, the bed is allowed to settle, and the position of valve V1 is changed to downward flow, valve V2 is opened and Valve V3 is closed, permitting the eluted rhApoA1 protein to be collected. This process could be further scaled to higher industrial levels, maintaining the same parameters.

5.4. CONCLUSION

Scale-up in downstream processing is always a major challenge in the global biopharma industry. While technology for the scaling up of production of recombinant proteins in microbes has advanced by major scales, protein purification is still a bottleneck, which contributes to over 80% of the drug manufacturing cost (OECD report). Thus, any improvement in the purification process has a direct impact on the cost-effective production of high added value proteins.

This chapter has successfully demonstrated the effective scale-up of production (to 5l benchtop bioreactor) and purification (in expanded bed adsorption mode) of rhApoA1 from *P. pastoris*. The adaptation of the purification process to work in expanded bed mode provides ample scope for incorporation into an industrial process for the direct capture of rhApoA1 directly from the production feed, without any sample pretreatment/clarification.

Chapter 6

Generation of ApoA1 variants: Milano & Paris

6.1. INTRODUCTION TO VARIANTS OF ApoA1

Over 50 naturally occurring mutations in ApoA1 have been reported with a natural missense or in-frame deletion mutations, resulting in reduced HDL-C levels and in certain cases amyloidosis (Matsunaga et al. 2010). The documented variations with their reported pathophysiological effects have been detailed in Fig. 6.1.

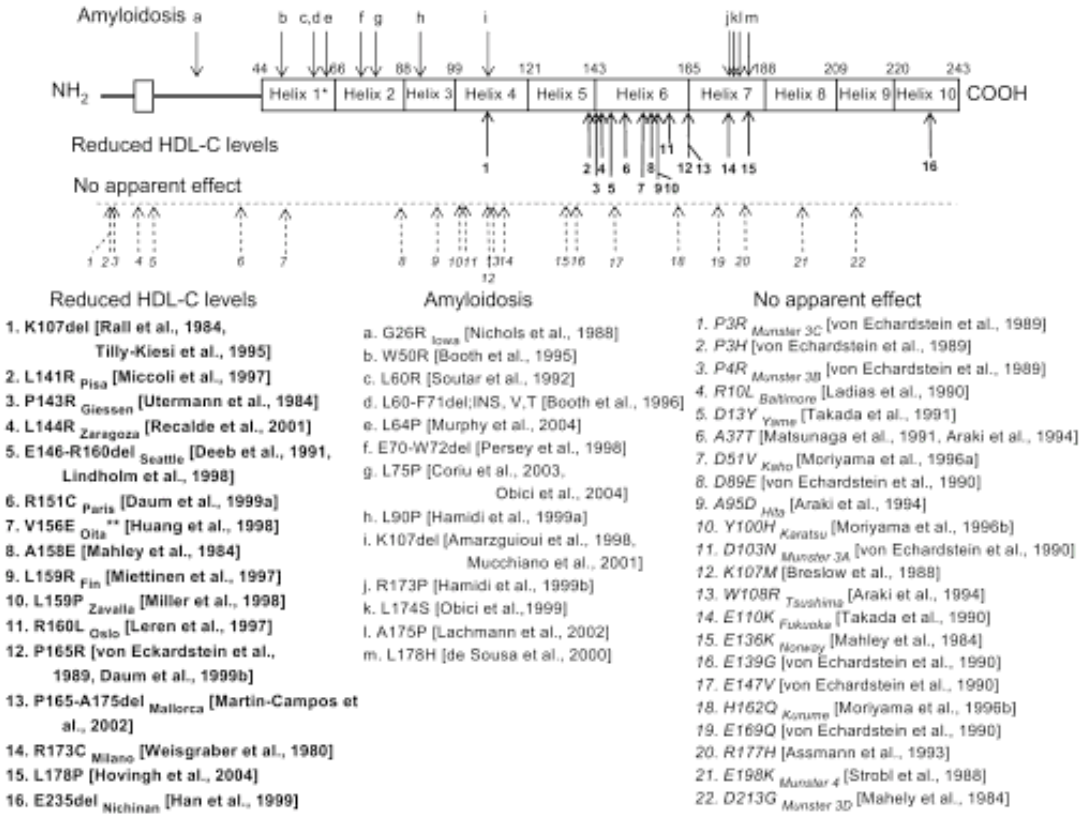


Figure 6.1. Naturally occurring ApoA1 missense mutations or in-frame deletions classified according to their pathophysiological outcomes.

Several of these mutations have been earlier discussed briefly in Section 1.3.4. In this chapter, the focus is on the Cysteine variants (Milano & Paris) of ApoA1.

6.1.1. IMPORTANCE OF ApoA1 MUTANTS: MILANO AND PARIS

$ApoA1_{Milano}$ and $ApoA1_{Paris}$ are both characterised by a single Arginine to Cysteine mutation at positions 173 and 151 respectively. Both variants have been reported in populations with reduced HDL-C levels but at the same time with reduced incidents of cardiovascular disorders. Wild type ApoA1 has no

Cysteine residues in its amino acid sequence (Brewer Jr. et al. 1978). The introduction of Cysteine in these two variants is hypothesised to increase stability of the resultant HDL molecule owing to homodimerization of ApoA1 (Klon et al. 2000).

6.1.2. STRUCTURAL CHANGES DUE TO POINT MUTATION AND POTENTIAL IMPACT ON FUNCTION

A few studies have investigated the effects of a single point Arginine to Cysteine mutation in ApoA1. An *in vivo* evaluation of $ApoA1_{Milano}$ revealed that both the loss of Arginine as well as the addition of Cysteine contribute to its altered function (Alexander et al. 2009). Arginine-173 of wild type ApoA1 is potentially involved in an inter-helical salt bridge with Glutamate-169, which is disrupted in the Milano variant, thereby destabilising the helix bundle in turn modifying its lipid binding characteristics. However, the exact mechanism by which the Cysteine variants are of better quality isn't yet clear. Hence, in this thesis work, the two variants, $ApoA1_{Milano}$ and $ApoA1_{Paris}$ were generated to support future functional studies to better elucidate the mechanism of action of these variants.

6.2. EXPERIMENTAL

6.2.1. MATERIALS

Custom oligonucleotides for introducing site directed mutagenesis were synthesised from Eurogentec (Agners, France). Competent *E. coli* DH5α cells (NEB5α) were purchased from New England Biolabs (Évry, France). All other chemicals for buffers and reagents were from Sigma-Aldrich (Saint-Quentin Fallavier, France).

6.2.2. SITE-DIRECTED MUTAGENESIS OF ApoA1 GENE TO GENERATE MILANO & PARIS CONSTRUCTS

Milano (R173C) and Paris (R151C) mutations were introduced by using the following sets of primers:

Milano-F: 5' ACGAGCTGCGCCAGTGCTTGGCCGC 3'

Milano-R: 5' GCGGCCAAGCACTGGCGCAGCTCGT 3'

Paris-F: 5' AGATGCGCGACTGCGCGCGCGCCCA 3'

Paris-R: 5' TGGGCGCGCGCGCAGTCGCGCATCT 3'

The mutagenesis was carried out using QuickChange® II Site-Directed Mutagenesis Kit (Agilent Technologies), as per the manufacturer's instructions. Briefly, a thermal cycling reaction was setup with template DNA (plasmid pPICZα-ApoA1), primers to incorporate mutations (Milano/Paris, as case may be), 10mM dNTPs and high-fidelity *Pfu* DNA polymerase and then subjected to thermal cycling: initial denaturation at 95ºC for 30secs, followed by 18 cycles of denaturation (95ºC, 30secs), annealing (55ºC, 1min), extension (72ºC, 5mins). Subsequent to the thermal cycling, the template strand was digested by *DpnI* treatment, and the amplified fragments were transformed into competentNEB5α cells and plated on LB-agar plates containing 50µg/ml Zeocin. Plasmids were then isolated from transformed *E. coli* cells and verified for mutations by DNA sequencing (Millegen, Labège, France).

6.2.3. TRANSFORMATION AND SCREENING OF rhApoA1-Milano & rhApoA1-Paris

After verification by DNA-sequencing, the constructs pPICZα-ApoA1-Milano and pPICZα-ApoA1-Paris were linearised by digestion with *PmeI*, and electroporated independently into competent *P. pastoris* X-33 cells as described earlier (Section 2.2.3). Subsequently, transforments were screened for their resistance to Zeocin up to 2mg/ml and verified for integration by colony PCR analysis as described earlier (Section 2.2.4).

6.2.4. FLASK-CULTURE EXPRESSION OF rhApoA1-Milano & rh-ApoA1-Paris

Three clones each of rhApoA1-Milano and rhApoA1-Paris were screened for their expression of the respective rhApoA1 mutant proteins. Single colonies of each clone were inoculated independently in 10ml BMGY containing 200µg/ml Zeocin as a preculture. After overnight growth at 30°C and 200rpm, the precultures were inoculated into 140ml of fresh BMGY (buffered glycerol complex media). After about 24hrs, when the cells had grown to a reasonable quantity (based on their OD at 600nm), they were centrifuged and resuspended in 150ml of BMMY (buffered methanol complex media). Induction was carried

out for a total of 5 days; with methanol being replenished every 24hrs at 0.5%. At the end of the induction, comparative day-wise expression of select clones along with the control were analysed by dot-blot analysis with ApoA1-specific polyclonal antibodies.

6.2.5. SCALE-UP OF EXPRESSION IN 2L BENCHTOP BIOREACTOR

One clone each from the pool of rhApoA1-Milano and rhApoA1-Paris were further subjected to expression studies in a 2l benchtop bioreactor.

First, a single colony was inoculated and grown in baffled flasks containing 100ml of buffered glycerol-complex medium (BMGY, 100mM potassium phosphate buffer, pH 6.0, 13.4g/L YNB, $4x10^{-4}$g/L biotin, 10g/L glycerol and 150 µg/mL Zeocin™) up to an OD (600nm) of 4-8, and this was inoculated in 2L of BMGY in a BIOSTAT® Bplus laboratory 2l bioreactor with a starting OD_{600} of about 0.3. The temperature and pH were maintained at 30°C and 6.0 respectively, and dissolved oxygen level was maintained at 15% saturation by regulating aeration and agitation in a cascade manner. After complete consumption of glycerol in the medium (verified by standard glycerol assay; data not shown), a methanol fed-batch phase was initiated by adding methanol every 12hrs to a final concentration of 0.5%. Samples were drawn every 24hrs to follow the growth profile and protein production. Protein production was monitored by SDS-PAGE analysis. After 120hrs of induction, the culture was harvested and the cell-free broth was frozen at -80°C.

6.2.6. PRELIMINARY PURIFICATION OF rhApoA1-Milano & rhApoA1-Paris

Purification of rhApoA1-Milano and rhApoA1-Paris were evaluated on two mixed-mode resins: HEA HyperCel (Pall Lifesciences) and CaptoMMC (GE Healthcare). The experimental conditions were similar to that optimised for the purification of wild type rhApoA1 (Section 3.3.2).

6.3. RESULTS

6.3.1. GENERATION OF rhApoA1-Milano & rhApoA1-Paris

The construct pPICZα-ApoA1 was subjected to site-directed mutagenesis as described in the experimental section, and the resulting clones (pPICZα-ApoA1-Milano and pPICZα-ApoA1-Paris) were verified by DNA sequencing. Upon transformation into competent *P. pastoris* X-33 cells, transformants were screened for their resistance to Zeocin up to 2mg/ml (Fig. 6.2).

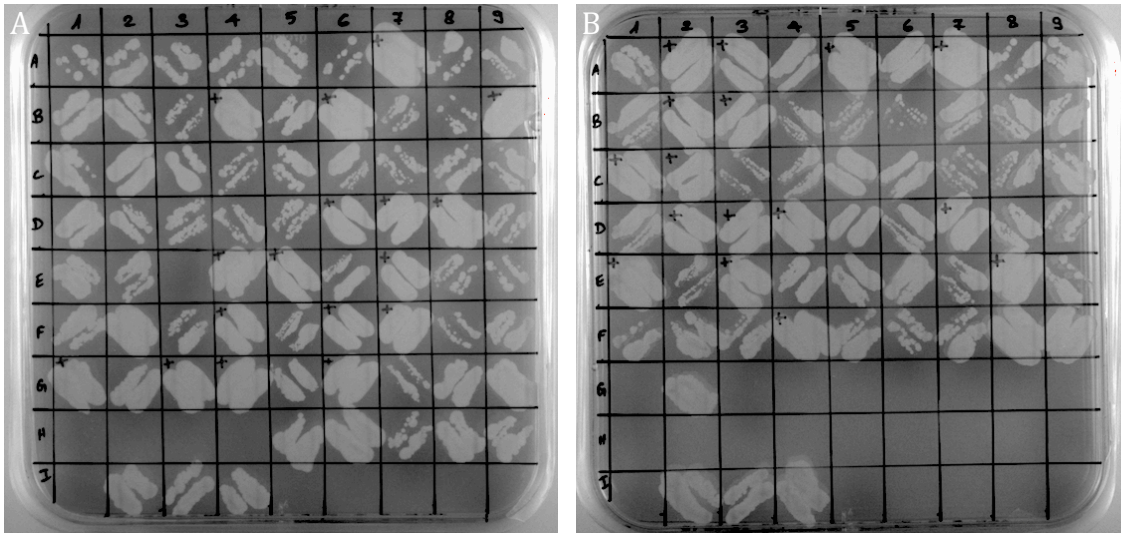


Figure 6.2. Screening of transformants of (a) rhApoA1-Milano and (b) rhApoA1-Paris on YPD plates containing 2mg/ml Zeocin.

Clones that showed consistent resistance to 2mg/ml Zeocin were further verified for integration of the mutated ApoA1 gene by colony PCR analysis (Fig. 6.3).

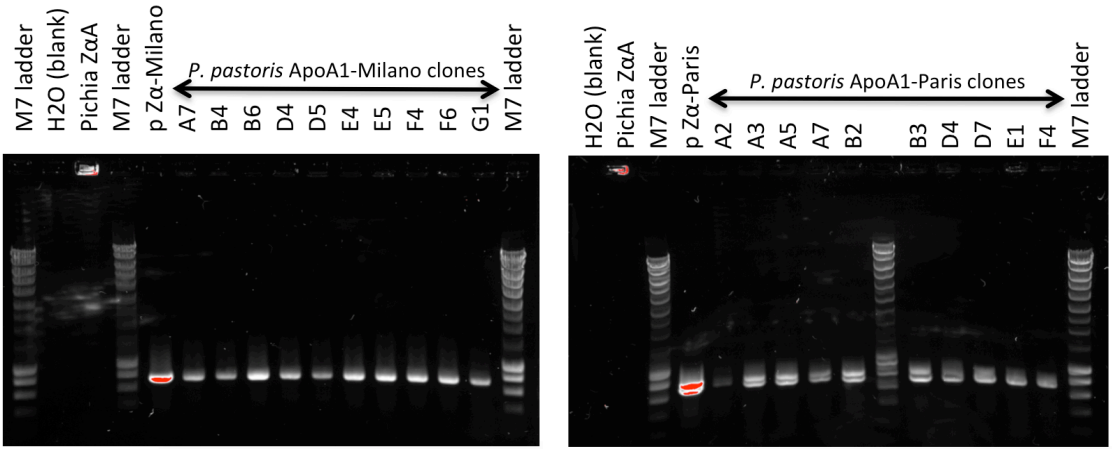


Figure 6.3. Colony PCR analysis of *P. pastoris* transformants with (a) rhApoA1-Milano and (b) rhApoA1-Paris constructs

Three colonies each from Milano and Paris transformants were further screened for preliminary expression studies in shake flasks.

6.3.2. FLASK-CULTURE EXPRESSION OF rhApoA1-Milano & rh-ApoA1-Paris

Three colonies transformed with ApoA1-Milano gene (A7, B6, E4) and three colonies transformed with ApoA1-Paris gene (A2, B3, E1) were subjected to preliminary expression studies in shake flasks, along with a vector transformed *P. pastoris* clone as a negative control (ZαA) as described in the methods section. The OD_{600nm} of cells were periodically monitored for growth, and samples drawn at various time points were analysed by dot-blot analysis using anti-ApoA1 polyclonal antibodies.

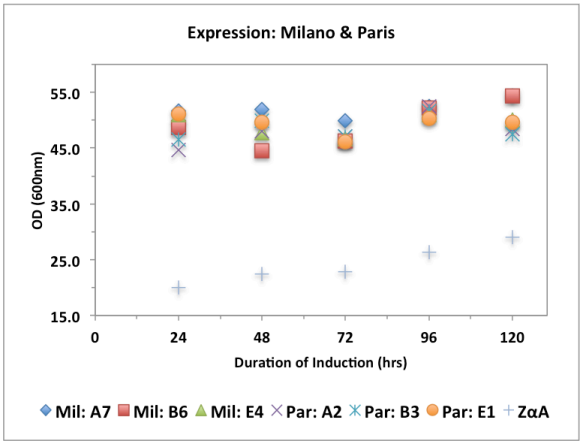


Figure 6.4. Growth profile (OD_{600nm}) of Milano & Paris clones on shake flask cultures

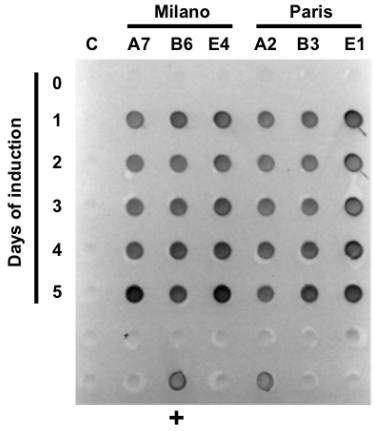


Figure 6.5. Dot-blot analysis of Milano & Paris clones upon five days of induction

All six clones exhibited consistent growth as evidenced by the absorbance at 600nm (Fig. 6.4). Production of rhApoA1 variants was confirmed by dot-blot analysis (Fig. 6.5), with an absence of signal prior to induction and with a constant rise in intensity of signal with increasing duration of induction. There was no signal corresponding to the vector-transformed X-33 cells (Fig 6.5 – lane C), verifying the specificity of the polyclonal antibodies used for the detection of ApoA1 and its variants. Purified wild type rhApoA1 (Fig. 6.5 – position '+') showed a clear signal, further validating the obtained results.

6.3.3. SCALE-UP OF EXPRESSION IN 2L BENCHTOP BIOREACTOR

Based on the expression profiles of the three Milano & three Paris cultures in shake flask experiments, one clone each (Milano: E4 & Paris: E1) were further scaled-up to be produced in a 2l benchtop bioreactor. The culturing conditions and parameters were similar to that optimised for the production of wild type rhApoA1, as explained in methods (Section 6.2.5). In both cases, the temperature and pH were maintained at 30ºC and 6.0, respectively. Dissolved oxygen levels were maintained at 15%, by a combination of agitation and aeration systems in a cascading manner. After the first 24hrs, and upon consumption of glycerol in the medium, induction was carried out by addition of 0.5% methanol every 12%. Samples were drawn at periodic intervals for analysis by 12% SDS-PAGE.

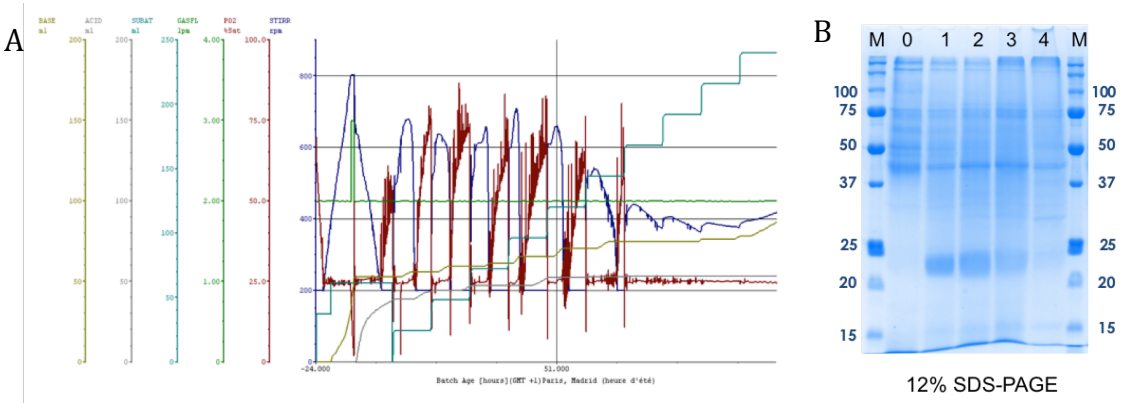


Figure 6.6. (a) Expression profile and (b) SDS-PAGE analysis of rhApoA1-Milano clone E4 on 2l benchtop bioreactor.

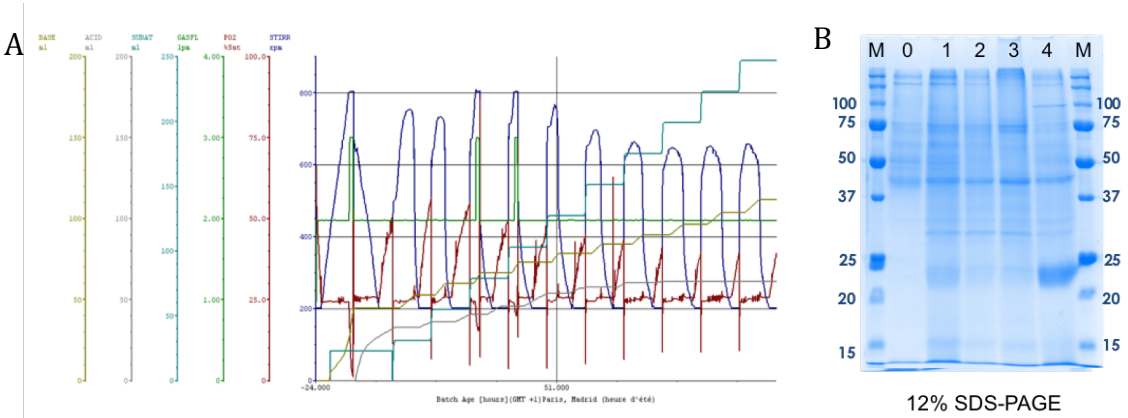


Figure 6.7. (a) Expression profile and (b) SDS-PAGE analysis of rhApoA1-Paris clone E1 on 2l benchtop bioreactor.

The rhApoA1-Milano clone E4 displayed consistent growth until day-3, after which there was a drop in agitation (Fig. 6.6(a)), indicating a reduced

metabolism of cells. This was substantiated by the reduced intensity of band upon 12% SDS-PAGE analysis (Fig. 6.6(b)), hinting reduced production levels and dilution of rhApoA1-milano content in the medium. Microscopic inspection of the cells on day 5 of expression showed no contamination, leading the reason for reduced metabolism and recombinant protein production towards other possible factors (more rapid substrate consumption, etc).

The rhApoA1-Paris clone E1, on the other hand displayed a model profile (Fig. 6.7(a)), with complete consumption of substrate every 12hrs, indicated by the rise in agitation and minor increases in dissolved oxygen in every 12-hour period. This was also supported by the corresponding 12% SDS-PAGE analysis, revealing maximum production on day 4 of induction (Fig. 6.7(b)).

The two batches of production confirmed the successful scale-up of production of rhApoA1-Milano and rhApoA1-Paris. Subsequently, purification of these two proteins were attempted based on optimised methods for wild type rhApoA1.

6.3.4. BINDING PATTERNS OF rhApoA1-Milano

Recovery of rhApoA1-Milano was first attempted with HEA HyperCel, using the conditions optimised earlier for wild type rhApoA1. Raising the pH of the *P. pastoris* expression broth containing rhApoA1-Milano caused precipitation of a fraction of the target protein (Fig. 6.8(b), lane 'ppt').

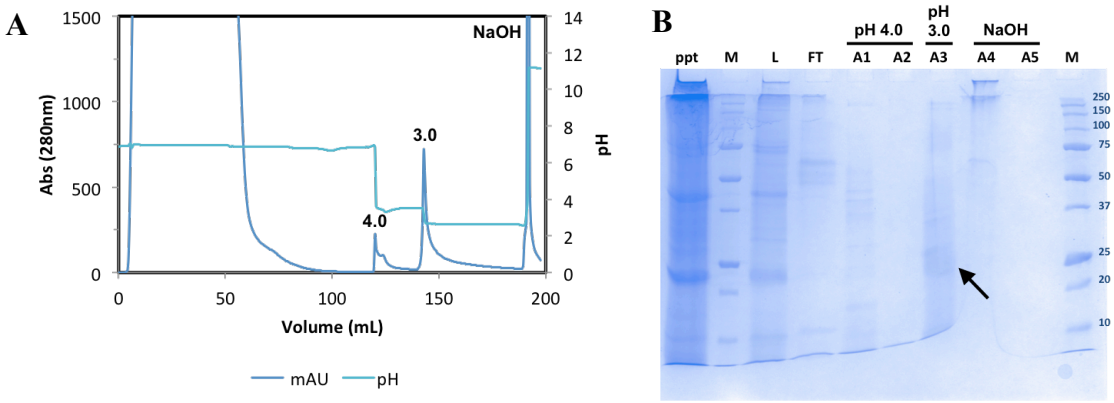


Figure 6.8. Purification of rhApoA1-Milano by HEA HyperCel. (a) Chromatogram and (b) 12% SDS-PAGE analysis of peak fractions

The chromatographic profile obtained with rhApoA1-Milano (Fig. 6.8(a)) was similar to that of wild type rhApoA1. Nevertheless, the protein (rhApoA1-Milano) was more strongly bound and was eluted only at pH 3.0 (Fig. 6.8(b), lane A3). This stronger binding does indicate towards an altered conformation of the folded rhApoA1-Milano protein with increased hydrophobicity, vis-à-vis its wild type counterpart. Although the target protein was recovered in part by elution at pH 3.0, there were several other protein bands present in the lane (Fig. 6.8(b), lane A3) which required further polishing prior to recovering the protein.

Secondly, the mixed mode resin CaptoMMC was evaluated for the recovery of rhAPoA1-Milano. Binding for all CaptoMMC experiments were at pH 4.5. It is well documented that both pH and conductive strength of the microenvironment strongly dictate binding to multi-modal ligands (Pezzini et al. 2015). Hence, in this study, two strategies were tested for the recovery of rhApoA1-Milano from *P. pastoris* expression broth: (i) by raising the pH while maintaining conductivity, followed by a rise in conductivity; and (ii) by eliminating the conductivity, followed by a rise in pH.

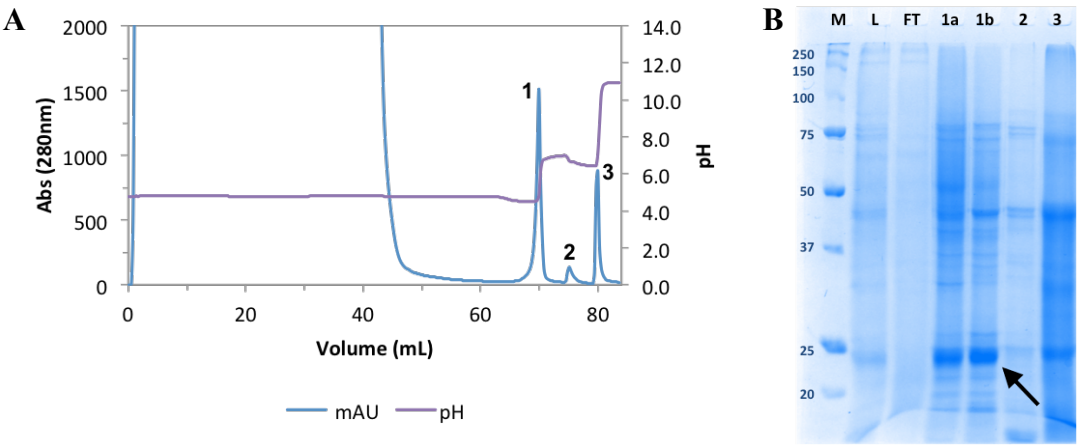


Figure 6.9. Purification of rhApoA1-Milano by CaptoMMC. (a) Chromatogram and (b) 12% SDS-PAGE analysis of load (L), non-retained (FT), and peak fractions at pH 7.0 + 250mM NaCl (#1), pH 7.0 + 2M NaCl (#2) & NaOH wash (#3).

By the first approach, the bound rhApoA1-Milano was efficiently recovered by raising the pH without eliminating the salt (Fig. 6.9, peak #1). However, a number of additional bands were present, which required further polishing in order to obtain our target protein in a pure form. A second concern was the

strong binding of a population of rhApoA1-Milano to the resin, which was eliminated only upon regeneration with 0.5M NaOH (Fig. 6.9, peak #3).

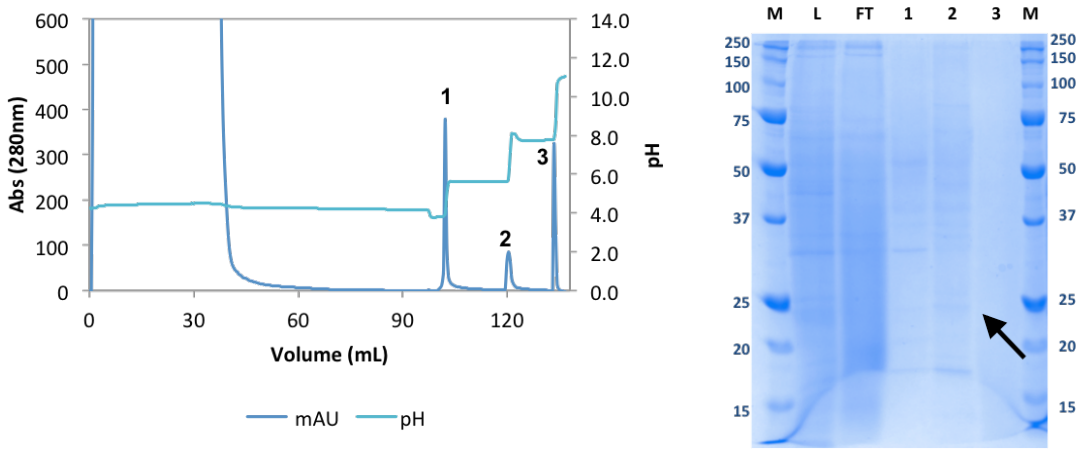


Figure 6.10. Purification of rhApoA1-Milano by CaptoMMC. (a) Chromatogram and (b) 12% SDS-PAGE analysis of load (L), non-retained (FT), and peak fractions at pH 7.0 (#1), pH 8.5 (#2) & NaOH wash (#3).

In the second approach (Fig. 6.10), the elimination of salt did not support the consistent binding of rhApoA1-Milano to the CaptoMMC resin, evidenced by the loss of certain amount of protein in the non-retained fraction (Fig. 6.10(b), Fraction 'FT'). As observed with the first approach, there was a small fraction of the target protein which bound stronger to the resin, evidenced by a faint signal in the elution fraction at pH 8.5 (Fig. 6.10, Fraction #2).

The differences in binding patterns of rhApoA1-Milano vis-à-vis wild type rhApoA1 to both HEA HyperCel and CaptoMMC resins strongly hint towards an increased hydrophobicity of rhApoA1-Milano. The stronger binding in HEA HyperCel, and the salt-dependent binding with CaptoMMC are both indicative of hydrophobic interactions, of an increased strength in comparison to the wild type rhApoA1 protein. A second factor to note is the presence of two populations of rhApoA1-Milano, one exhibiting weak interactions with CaptoMMC and the other of relatively stronger nature. It would be of interest to compare the functional activities of both fractions of rhApoA1-Milano to better understand their folding-related functional changes. Nevertheless, further polishing is required to recover the rhApoA1-Milano protein in its purified form for further analysis.

6.3.5. BINDING PATTERNS OF rhApoA1-Paris

The binding profile of rhApoA1-Paris was evaluated on both HEA HyperCel and CaptoMMC chromatographic ligands.

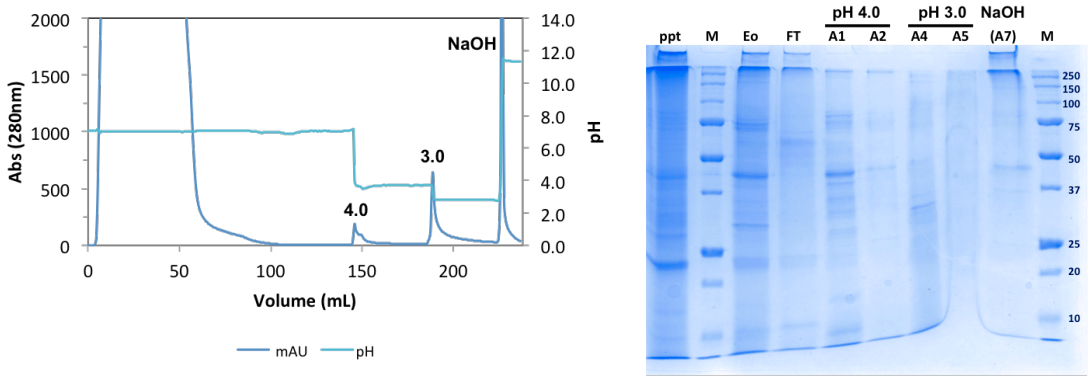


Figure 6.11. Purification of rhApoA1-Paris by HEA HyperCel. (a) Chromatogram and (b) 12% SDS-PAGE analysis of load, non-retained and peak fractions.

As observed earlier with rhApoA1-Milano, raising the pH of the *P. pastoris* expression broth containing the rhAPoA1-Paris protein formed a precipitate (Fig. 6.11(b), lane 'ppt'), containing the target protein. However, most of the rhApoA1-Paris present in the load was washed off in the non-retained fraction, and a small amount of bound rhApoA1-Paris was recovered at pH 4.0 (Fig. 6.11(b), lane 'A1').

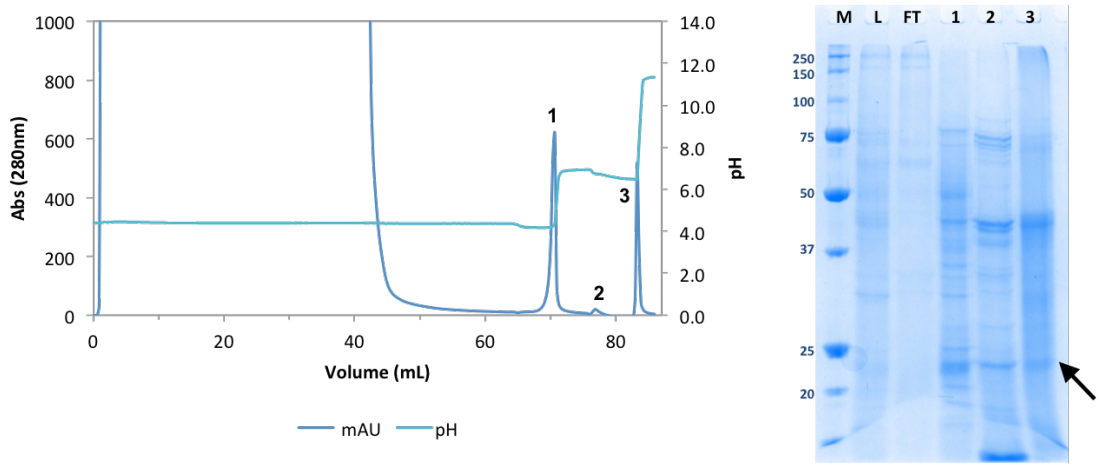


Figure 6.12. Purification of rhApoA1-Paris by CaptoMMC. (a) Chromatogram and (b) 12% SDS-PAGE analysis of load (L), non-retained (FT), and peak fractions at pH 7.0 + 250mM NaCl (#1), pH 7.0 + 2M NaCl (#2) & NaOH wash (#3).

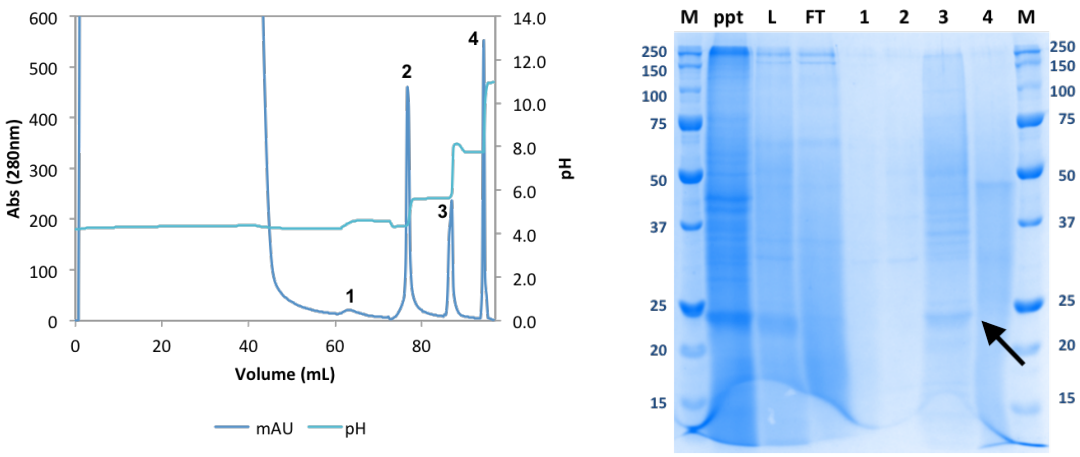


Figure 6.13. Purification of rhApoA1-Paris by CaptoMMC. (a) Chromatogram and (b) 12% SDS-PAGE analysis of load (L), non-retained (FT), and peak fractions at pH 5.0 (#1), pH 7.0 (#2), pH 8.5 (#3) & NaOH wash (#4).

While evaluating CaptoMMC for the capture of rhApoA1-Paris, two strategies were envisaged (similar to previous section). Binding was carried out at pH 4.5, with 250mM NaCl (moderate conductivity) in all cases. The following two elution strategies were evaluated: (i) rise in pH and subsequently conductivity; and (ii) elimination of salt followed by a rise in pH.

By the first strategy, all rhApoA1-Paris in the *P. pastoris* expression broth was captured by the ligand, however, with varying degrees of binding. This was substantiated by the presence of the bound rhApoA1-Paris in all elution fractions (Fig. 6.12(b)). Yet again, numerous other bands were present in each elution fraction demanding further polishing.

The alternate strategy of eliminating salt and subsequently rising the pH proved to be more effective in the recovery of rhApoA1-Paris from the *P. pastoris* expression broth. The bound protein was recovered in the elution step at pH 8.5, again indicating a stronger binding to CaptoMMC than wild type rhApoA1.

Despite the intriguing chromatographic profiles of rhApoA1-Paris on both HEA HyperCel and CaptoMMC, further polishing is required to recover the target protein in its pure form.

6.4. CONCLUSION

In this chapter, the two Cysteine variants of ApoA1: Milano and Paris have been successfully generated. There was a two-fold objective in producing these variants:

(i) Generation of reagents for further functional comparison with wild type ApoA1

(ii) Setting up a process for production of rhApoA1-Milano and/or rhApoA1-Paris for potential therapeutic applications

The expression profiles and scale-up of expression of the two recombinant variants were successful, and similar to that obtained with wild type rhApoA1. Consistent growth and production was observed in the case of both variants, as validated by dot-blot and SDS-PAGE analyses.

Despite minor differences in their sequences (single point Cysteine variants), this work has reported a significant difference in binding profiles of both rhApoA1-Milano and rhApoA1-Paris to the mixed-mode ligands HEA HyperCel and CaptoMMC. The rhApoA1-Milano exhibited an enhanced hydrophobicity, as evidenced by its stronger retention on the HEA HyperCel ligand. Furthermore, the binding of all three proteins – wild type, Milano & Paris variants of ApoA1 seem to be defined by local interactions of exposed hydrophobic pockets with the mixed-mode ligands, which are defined by the pH and conductivity of the surrounding conditions.

Both variants (rhApoA1-Milano and rhApoA1-Paris) require further polishing to recover them in homogeneity for future functional analyses. The bioprocess for the production of these two variants could pave way for a more efficient method for producing them for eventual therapeutic applications.

Chapter 7

General Conclusions and Perspectives

This thesis has worked towards the development of a method for efficiently producing wild type and variants of human apolipoprotein A-I in *P. pastoris*. Production of recombinant ApoA1 has been previously described using different systems. The bacterial system *E coli* presented the disadvantage of instability of mRNA and rapid degradation of mature ApoA1 (Isacchi et al. 1989). Another disadvantage was the use of tags to facilitate purification steps (Schmidt et al. 1997; Bergeron et al. 1997): despite the presence of these tags, the purification steps require many treatment such as cell lysis, protein precipitation with ammonium sulphate, delipidation, endotoxin clearance at the end of the purification, in addition to an enzymatic cleavage of the affinity tag. In some cases the protein is mainly found into inclusion bodies, which further complicates the purification strategy (Angarita et al. 2014).

Another reported approach was the production of Apolipoprotein A-I using baculovirus-insect cell expression system (Moguilevsky et al. 1994). In this case, it was necessary to express it in the proapolipoprotein form in order to ensure the secretion of the protein. Without the proapoA-I sequence, most of the mature apolipoprotein A-I was found in the cytoplasm and only a small proportion of ApoA-I was secreted into the culture medium.

The production of Apolipoprotein A-I has also been evaluated in Chinese Hamster Ovary Cells (Schmidt et al. 1997). However, typical yields were only 0.5 to 1 μg/mL Apolipoprotein A-I was secreted by CHO in a serum-free culture medium, and upto 10 μg/mL after several optimisation. Higher concentrations were also achieved, however, immunoblot analysis revealed a carboxy-Terminal proteolysis.

Few studies have used the *Pichia pastoris* expression system to produce apolipoprotein A-I (Feng et al. 2006). In the present study, the focus was on the production of apolipoprotein A-I exploiting *P. pastoris*' ability to produce and secrete recombinant proteins with high yield. A high expressing clone was selected after screening with zeocin, and production yields of 20 mg/L was

achieved upon optimization of methanol induction phase. This production is significantly higher than that reported in CHO cells.

For any production/purification process to be industrially viable, the focus is always on minimising the number of processing steps while maximising recovery/yield. In this study, the rhApoA1 has been expressed without the addition of any affinity tag so that it may be generated close to its native conditions, in turn reducing the number of post-processing steps like proteolytic cleavage to remove tags, etc.

Eventually, a scalable two-step method has been developed: using mixed-mode chromatography to capture the expressed rhApoA1 in continuous mode without any pre-treatment or clarification followed by an anion exchange polishing step, which demonstrates greater advantages in terms of efficiency and number of processing steps in comparison to the previously published 12-step method with lower scalability (Feng et al. 2006).

In addition, two naturally occurring variants of rhApoA1 (Milano and Paris) have been generated and produced in 2 litre benchtop bioreactors. Further optimisation and process development is required before scaling up to produce them as therapeutic proteins.

After the generation of the variants of rhApoA1, comprehensive comparative studies (biochemical and *in vivo*) would help understand better the mechanisms by which the Milano and Paris variants are more protective. In conclusion, this thesis has worked towards setting a foundation for further developmental work to be done in order to generate therapeutic ApoA1 molecules.

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