# Influence of tryptophan tags on the purification of cutinase, secreted by a recombinant *Saccharomyces cerevisiae*, using cationic expanded bed adsorption and hydrophobic interaction chromatography

**M. E. Lienqueo · O. Salazar · C. R. C. Calado · L. P. Fonseca · J. M. S. Cabral**

**Abstract** During cationic bed adsorption (EBA), with cutinase with varying length tryptophan tags $(WP)_2$ and $(WP)_4$, 33% and 10% of adsorption capacity and 80% and 32% eluted specific activity were observed in relation to wild type (wt)-cutinase in the conventional process. Therefore, as the hydrophobicity of the protein increases, it is important to integrate the EBA step with a hydrophobic interaction chromatography (HIC) process. As the length of the hydrophobic tag-(WP) increases from $n = 2$ to $n = 4$, the purification factor obtained by HIC was 1.8 and 2.2-fold higher than wt-cutinase. However, the recovery yield obtained in HIC decreases substantially as the length of hydrophobic tag increases (97%, 84% and 70% for wt-cutinase, cutinase-$(WP)_2$ and cutinase-$(WP)_4$). The integration of two purification steps, EBA followed by HIC, resulted in the highest overall purity level for cutinase-$(WP)_2$, and the highest overall recovery yield for wt-cutinase. When optimizing the design of a hydrophobic tag fused to a protein secreted by *Saccharomyces cerevisiae* it must be considered that the cultivation parameters could impair the downstream process, and consequently the optimum tag is not necessarily the one that presents the highest purification factor in HIC.

**Keywords** Cutinase · Expanded bed adsorption · Hydrophobic interaction chromatographic · Optimal hydrophobic tag design

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## Introduction

Today, the limiting stages in recombinant protein production are no longer only the strain selection or fermentation stages but much more those of recovery and purification (i.e. downstream processing, DSP), so most of the research efforts are focused on DSP optimization and its integration to fermentation processes. To enhance downstream processing, protein engineering can be used due to its ability to confer particular properties to a protein, such as specific affinity, and change its charge or hydrophobicity. The most common modification involves the fusion of peptide tags to the protein (Terpe 2003). Then, the purity factor in a purification process for a

target protein can be increased and considerable improvements in yields and costs of downstream purification processes can be achieved with the use of such tags (Steffens et al. 2000).

The addition of hydrophobic tags is efficient in hydrophobic interaction chromatography (HIC) where the proteins can be eluted with different retention times (Hassinen et al. 1994; Kepka et al. 2005). This is a more cost-efficient chromatographic method for protein purification since HIC is less expensive compared to affinity chromatography based on ligands such IMAC, protein A and IgG. Some of the most commonly used are tryptophan-containing tags (e.g. $(WP)_2$, $(WP)_4$) (Bandmann et al. 2000; Berggren et al. 1999; Fexby and Bulow 2004; Kepka et al. 2005; Rodenbrock et al. 2000). Unfortunately, these short hydrophobic peptide tags could have some negative effects on the protein production and/or purification, for example: (a) hydrophobic fusion can cause the protein to be membrane-associated (Persson et al. 1988); (b) proteolytic cleavage can reduce the amount of protein obtained (Collen et al. 2001; Hassinen et al. 1994); (c), changes in mRNA stability that will affect the expression levels (Fexby and Bulow 2004); (d) increased hydrophobic properties might result in protein association of dimers or larger aggregates (the tagged protein is partly/fully insoluble) (Sagt et al. 1998), or in dissociation into smaller subunits (Johansson and Walter 2000); (e) the conformational changes might lead to denaturation and collapse of the protein stability loss (Terpe 2003).

There are some studies about the effect of the production and recovery of fusion cutinases (Calado et al. 2002a; Cunha et al. 2003; Kepka et al. 2005; Sagt et al. 1998). In order to obtain an efficient and low cost production system for cutinase from *Fusarium solani pisi* (wild-type cutinase), this enzyme can be overproduced in *Saccharomyces cerevisiae*. The secreted wild-type cutinase could be recovered directly from the unclarified broth by expanded bed adsorption (EBA) (Calado et al. 2001, 2004) However, several studies showed that the increased hydrophobicity of cutinase can lead to impaired protein secretion in *S. cerevisiae* (Calado et al. 2002a; Sagt et al. 1998). Furthermore, it was observed that the fusion of hydrophobic peptides, (WP)2 or (WP)4 to wild-type cutinase changes the physical properties of the fused proteins, limiting cutinase secretion and subsequently leading to a lower plasmid stability, lower yeast cell growth rate, lower cutinase production per biomass and, consequently, lower extracellular cutinase activity, that consequently could impair the downstream process (Cunha et al. 2003).

The aim of this study was to evaluate quantitatively the effect of the fused $(WP)_2$ and $(WP)_4$ peptides on the host yeast growth and cutinase recovery and purification process by expanded bed adsorption and subsequent purification by HIC, and define criteria for selecting the best hydrophobic tags.

## Materials and methods

### Microorganism

The wild type (wt) cutinase, cutinase-$(WP)_2$ (fusion peptide composed of two tryptophan residues interspersed with two proline residues) and cutinase-$(WP)_4$ (fusion peptide composed of four tryptophan residues interspersed with four proline residues) producing *Saccharomyces cerevisiae* MM01 strains (Mata, leu2-3, ura3, gal1: URA3, MAL-8, MAL3, SUC3), containing the expression vectors pUR7320, pUR807, and pUR806, respectively, were constructed and provided by Unilever Research Laboratory, Vlaardingen, The Netherlands.

### Cultivation and cell harvesting

Cultivation, cell harvesting and activity assay were carried out as described in Calado et al. (2002b).

### Expanded bed adsorption (EBA)

Adsorption experiments were carried in a 45 cm glass column with 1.4 cm diameter with a settled bed adsorbent height of 14.0 cm. The fluid was distributed at the column inlet by a simple stainless steel basal mesh (40 µm). The cationic adsorbent Streamline SP XL (Amersham Pharmacia Biotech, Sweden) was used. The adsorbent was equilibrated and washed with 20 mmol/l citric buffer pH 4.5. During the expanded bed operation a constant flow velocity of 270 cm/h was maintained. Following bed expansion stabilization and adsorbent buffer equilibration, the whole broth previously adjusted at pH 4.5 with 1 M HCl was applied to the column. The reversible

deactivation of cutinase that may occur at pH 4.5 was taken into account when estimating the cutinase dynamic adsorption capacity. Frontal feedstock adsorption is applied until 5% of cutinase activity is detected at the column outlet in relation to cutinase activity at the column inlet. After that, broth recirculation is started until a constant cutinase activity is observed at the culture broth. The is subsequently washed until the effluent was devoid of yeast cells. The elution was carried out using the same column in downward flow, using a constant flow velocity of 122 cm/h with the starting buffer containing 500 mM NaCl. The cutinase activity and protein concentration of the column effluent fractions collected were analyzed.

### Hydrophobic interaction chromatography (HIC)

HIC was performed in a Pharmacia Fast Protein Liquid Chromatography (FPLC) system equipped with a 500 µl injection loop. The chromatographic column was 1 ml butyl Sepharose 6FF. The experiments were performed at room temperature, using a flow rate equal to 0.75 ml/min and 10 column volumes. Elution was obtained by a decreasing concentration gradient of analytical reagent grade ammonium sulphate. The initial eluent was 20 mM Bis–Tris, pH 7.0, plus 2 M ammonium sulphate. The final eluent was 20 mM Bis–Tris, pH 7.0). Fractions were collected and the enzyme activity and protein concentration were measured over the entire chromatogram.

### Analytical methods

Analytical methods for determining biomass, plasmid stability, cell viability, protein concentration, cutinase activity assay and enzyme secretion efficiency were carried out as described in (Calado et al. 2002b).

## Results and discussion

### Cultivation process

The cultivation parameters obtained after 72 h of *S. cerevisae* growth in batch mode are presented in Table 1. It was observed that the increase of the hydrophobic peptide tag $(WP)_n$ length resulted in a relevant cutinase retention in the yeast host-cell, that leads to both plasmid stability and yeast growth rate decrease, as observed previously by other authors (Calado et al. 2002a; Sagt et al. 1998). A main consequence of the increased hydrophobic peptide length (WP), was a decrease in cutinase activity, being observed 41, 22 and 2 U/ml, and cutinase specific activity, of 350, 200 and 40 U/mg, to the extracellular medium of the wt-cutinase, cutinase-$(WP)_2$ and cutinase-$(WP)_4$, respectively.

**Table 1** Cultivation parameters obtained after 72 h of *S. cerevisae* cultivation

| Cutinase produced by the yeast strain | wt-cutinase | Cutinase-$(WP)_2$ | Cutinase-$(WP)_4$ |
|---|---|---|---|
| Biomass (g dcw/l) | 8.5 | 7.9 | 6.9 |
| Plasmid stability (%) | 92 | 80 | 65 |
| Cell viability (%) | 97 | 96 | 97 |
| Secretion efficiency (%) | 72 | 60 | 25 |
| Cutinase activity (U/ml) | 41 | 22 | 2 |
| Cutinase specific activity (U/mg protein) | 350 | 200 | 40 |
| Cutinase specific cell activity (U/ mg dcw) | 4.8 | 2.8 | 0.3 |

dcw = dry cell wt

wt = wild-type

### Recovery of cutinases by expanded bed adsorption (EBA)

Secreted cutinase was recovered directly from the unclarified broth by expanded bed adsorption. In a previous work of Calado et al. (2002a), using the same wt-cutinase producing strain and the same media composition in batch mode, a Streamile-25 EBA column was loaded with whole broth previously diluted with water at 4:5. This dilution reduced both the media conductivity and the cell concentration, and consequently led to an increase in the dynamic cutinase adsorption to the cationic exchanger in relation to a similar experiment using media broth less diluted with water, being obtained 400 U/ml adsorbent. The reduction of the cell concentration in the culture that passes through the cationic EBA column in conditions such as the present ones (pH 4.5) is very important. Cells at such a low operational pH interact with each other, and with the adsorbent, reducing the total adsorbed cutinase by two different mechanisms: (i) direct competition with cutinase

towards the adsorbent; (ii) by perturbing the plug-flow in the EBA column due to formation of large cell aggregates that results in preferential channeling in the column.

In order to increase further the dynamic cutinase adsorption capacity to the cationic exchanger in relation to the Calado et al (2002a) work, in the present manuscript, a higher dilution degree of whole broth (at 1:2 with water) was used. Furthermore, the diluted culture broth was adsorbed by frontal application until 5% cutinase activity was reached at the column outlet in relation to cutinase activity at the column inlet, followed by broth recirculation until a constant cutinase activity was observed in the culture broth. In this way, a 6.1 fold-higher dynamic adsorption capacity of the cationic resin was achieved, however, at the expense of a lower purification capacity. Calado et al. (2002b) obtained an eluted cutinase specific activity similar to the one observed in a conventional purification process. In the present work, only 79% of the specific activity was observed in the eluted fraction in relation to the conventional purification process involving discrete sequences of fermentation, broth clarification by microfiltration, ultrafiltration and chromatographic purification as two fixed-bed, anion-exchange beds (Calado et al., 2001). In part, the lower purification factor could be due to the present use of a higher salt concentration in the elution step (500 mM NaCl) in relation to the salt concentration used by Calado et al. (2002a) (150 mM NaCl).

The previous EBA conditions were used to directly sequester cutinase-$(WP)_2$ and cutinase-$(WP)_4$. As the peptide tag $(WP)_n$ length increases a decrease of extracellular activity was observed, that resulted in dynamic adsorption capacities of 50% and 10% for cutinase-$(WP)_2$ and cutinase-$(WP)_4$, respectively, in relation to wt-cutinase. The reduction of the dynamic adsorption capacity depends directly on the cutinase concentration in the diluted culture broth, as the same ratio, between the dynamic adsorption capacity and the activity in the culture broth, of 27 or 28 was observed with the three recombinant systems, independently of the physico-chemical characteristics of the target protein. With wt-cutinase, a high concentration factor (8.5) and a high recovery yield (97%) was observed. The concentration factors obtained for the other two cutinase systems were also high, for cutinase-$(WP)_2$ and cutinase-$(WP)_4$ concentration factors of 8.7 and 3.8 for cutinase-$(WP)_2$ were obtained, respectively. However, as the hydrophobic peptide length increases the recovery yield significantly decreased, for cutinase-$(WP)_2$ and cutinase-$(WP)_4$, recovery yields of 79% and 62% were obtained (see Table 2).

As the length of the hydrophobic tag increases, the extracellular cutinase observed in the whole broth decreased: the enzyme activity recorded in the culture broth for the cutinase-$(WP)_2$ and cutinase-$(WP)_4$ were of 52% and 5% , in relation to wt-cutinase. As the major consequence, as the length of the peptide tag increased, higher is the proportion of the media proteins from the nitrogen-source and other proteins secreted by the yeast-host in relation to the target-protein. Therefore, the specific cutinase activity for the cutinase-$(WP)_2$ and cutinase-$(WP)_4$ were 57% and 11%, respectively, in relation to the wt-cutinase. In the EBA system, the purification factors observed were 1.5, 1.9 and 3.8 for wt-cutinase, cutinase-$(WP)_2$ and cutinase-$(WP)_4$, respectively, in relation to the broth specific activity. In the case of cutinase-$(WP)_2$, in spite of a higher purification factor, a similar specific activity (purity level) was obtained in relation to wt-cutinase. However, in relation to cutinase-$(WP)_4$, and in spite of the highest purification factor obtained (3.8), only 32% of the purity level obtained by an conventional purification process was achieved.

In resume, with the present EBA conditions, it was possible to significantly increase (by 6.1-fold) the dynamic adsorption capacity of the cationic-streamline resin, while maintaining a high recovery yield (of 97%) in relation to previous work performed by Calado et al. (2002a). However, this was achieved at the expense of a 20% lower purity level in relation to the previous EBA system. For the cutinase-$(WP)_2$ system, a similar 20% lower purity level in relation to a conventional purification system was achieved. Furthermore, a low recovery yield of 79% was observed. In the case of cutinase-$(WP)_4$, that is secreted at the lowest quantity to the extracellular medium, the lowest

**Table 2** Characteristic parameters of the EBA process

| Cutinase produced by the yeast strain | wt-cutinase | Cutinase-$(WP)_2$ | Cutinase-$(WP)_4$ |
|---|---|---|---|
| Dynamic adsorption capacity (mg cutinase/ml adsorbent) | 3.8 | 1.9 | 0.5 |
| Concentration factor | 8.5 | 8.7 | 3.8 |
| Recovery yield of cutinase (%) | 97 | 79 | 63 |
| Purification factor | 1.5 | 1.9 | 3.8 |

recovery yield (63%) was achieved in relation to the three recombinant systems evaluated. In this case, only 32% of the specific activity was obtained in relation to a conventional purification process. In all of the three cases, however, the present EBA, using a "laboratory made column" presents a highly valuable first downstream process for cutinase concentration by a factor between 4 and 9, and to obtain a purification factor between 1.5 and 3.8.

### Purification of cutinases by hydrophobic interaction chromatography (HIC)

In order to purify further the eluted cutinase obtained from the EBA process, a second purification step using HIC was conducted. The results of this step are summarized in Table 3. As the length of the fused tryptophan tag increases, the retention time of the target protein increases, resulting in purification factors of 1.8- and 2.2-fold higher for cutinase-$(WP)_2$ and cutinase-$(WP)_4$, respectively, in relation to wt-cutinase. However, the introduction of a peptide tag to the cutinase backbone resulted in recovery yield decreases, in a similar way as observed in the EBA process: for wt-cutinase, cutinase-$(WP)_2$ and cutinase-$(WP)_4$ recovery yields of 94%, 67% and 70% were observed. This last observation, in both purification steps (EBA and HIC) could be due to a possible protein association between dimers and/or larger aggregates (Johansson and Walter 2000).

**Table 3** Characteristic parameters of HIC purification on butyl Sepharose 6FF 2 M ammonium sulphate and overall process (EBA + HIC)

| Cutinase produced by the yeast strain | wt-cutinase | Cutinase-$(WP)_2$ | Cutinase-$(WP)_4$ |
|---|---|---|---|
| *HIC purification* | | | |
| Retention time (min) | 19.7 | 20.3 | 21.7 |
| Concentration factor | 2.2 | 2.0 | 1.7 |
| Recovery yield of cutinase (%) | 97 | 84 | 70 |
| Purification factor | 4.4 | 8.1 | 9.5 |
| *Overall process (EBA + HIC)* | | | |
| Overall recovery yield of cutinase (%) | 94 | 67 | 44 |
| Overall Concentration factor | 18.8 | 17.3 | 6.4 |
| Overall Purification factor | 6.5 | 15.2 | 35.9 |

Additionally, the concentration factor decreases with the length of the fused tryptophan tags.

## General observations and conclusions

With a purification process, that includes cationic Expanded Bed Adsorption (EBA) followed by hydrophobic interaction chromatography (HIC), it was possible to purify 3 cutinases: one wild-type (wt-) cutinase, and two cutinase-proteins fused with the hydrophobic tags $(WP)_2$, and $(WP)_4$, directly from crude feedstock. In the cultivation step, cellular growth, stability of plasmids, secretion efficiency of proteins and cutinase activities were negatively affected by the increase of the length of the peptide $(WP)_n$ fused to wt-cutinase. In the recovery and concentration step performed by EBA, the adsorption capacity and recovery yield decreased with the increase in the length of the peptide $(WP)_n$ fused to wt-cutinase, while the purification factor increased with the length of the tryptophan fused to wt-cutinase. In the present conditions, it was possible to obtain a much higher dynamic adsorption capacity towards cutinase in relation to other authors; however this was possible only at the expense of a decrease in purification capacity. Therefore, to further purify cutinase, it was necessary to apply a second purification process, hydrophobic interaction chromatography (HIC).

As the length of the hydrophobic tag increases, the retention time of cutinase in the hydrophobic chromatography column also increases, resulting in an increased purification capacity in relation to wt-cutinase. In both purification systems (EBA and HIC), the reduction in the recovery yield observed could result from increased cutinase aggregation as the hydrophobicity of the fused tag increases. Therefore, to purify hydrophobic-tagged cutinase, if the goal is to obtain the highest purity level at the expense of a lower recovery yield, both EBA and HIC processes must be performed. If the goal is only to obtain a concentrated protein, presenting a purity level between 0.2 and 0.8 in relation to the specific activity obtained by a conventional purification system, it will be enough to apply the EBA process.

At the overall purification end (that is EBA + HIC), the purification factor increases with the tag length, being observed a 2.3- and 5.5-fold higher purification factor for cutinase-$(WP)_2$ and cutinase-

$(WP)_4$, respectively, in relation to wt-cutinase. However, since as length of the peptide tag increases, the cutinase secretion to the extracellular medium will also be proportionally impaired, that consequently results in a substantially decreased purity levels of the target protein in the culture broth, being obtained a specific activity of 0.57% and 11% for cutinase-$(WP)_2$ and cutinase-$(WP)_4$, respectively, in relation to wt-cutinase. Therefore, as the length of the hydrophobic tag increases, the purification factor increases but the purity level does not necessary increases, since the final purity level is highly affected by the specific activity at the start of the purification process. With the present 3 cutinase systems, with an overall purification process that includes EBA and HIC, the highest purification factor was observed with cutinase-$(WP)_4$, but the highest purity level was reached with cutinase-$(WP)_2$. With cutinase-$(WP)_4$ a purification factor 2.4-fold higher was observed in relation to cutinase-$(WP)_2$. However, with cutinase-$(WP)_2$, a purity level of 2.1-fold higher was reached in relation to the $(WP)_4$ system. Another important factor, is the recovery yield in the purification steps that significantly decreases with the increased length of the hydrophobic tag, with the increased length of $-(W)_2$ for $-(WP)_4$ an decrease of 14% on the overall recovery yield was observed. Therefore, the fusion protein with the hydrophobic $-(WP)_2$ tag proved to be the best candidate that will imply the highest purity level. The present manuscript also put in evidence the importance of considering the effect of the cultivation parameters on the efficiency of the purification step, as lower extracellular cutinase activity and specific activity, could impair the downstream process.

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## Reference


Bandmann N, Collet E, Leijen J et al (2000) Genetic engineering of the Fusarium solani pisi lipase cutinase for enhanced partitioning in PEG-phosphate aqueous two-phase systems. J Biotechnol 79:161–172

Berggren K, Veide A, Nygren PA et al (1999) Genetic engineering of protein-peptide fusions for control of protein partitioning in thermoseparating aqueous two-phase systems. Biotechnol Bioeng 62:135–144

Calado CR, Hamilton GE, Cabral JM et al (2001) Direct product sequestration of a recombinant cutinase from batch fermentations of *Saccharomyces cerevisiae*. Bioseparation 10:87–97

Calado CR, Cabral JM, Fonseca LP. (2002a) Effect of *Saccharomyces cerevisiae* fermentation conditions on expanded bed adsorption of heterologous cutinase. J Chem Technol Biotechnol 77:1231–1237

Calado CRC, Taipa MA, Cabral JMS et al (2002b) Optimisation of culture conditions and characterisation of cutinase produced by recombinant *Saccharomyces cerevisiae*. Enzyme Microb Technol 31:161

Calado CR, Ferreira BS, da Fonseca MM et al (2004) Integration of the production and the purification processes of cutinase secreted by a recombinant *Saccharomyces cerevisiae* SU50 strain. J Biotechnol 109:147–158

Collen A, Ward M, Tjerneld F et al (2001) Genetic engineering of the Trichoderma reesei endoglucanase I (Cel7B) for enhanced partitioning in aqueous two-phase systems containing thermoseparating ethylene oxide–propylene oxide copolymers. J Biotechnol 87:179–191

Cunha MT, Costa MJ, Calado CR et al (2003) Integration of production and aqueous two-phase systems extraction of extracellular Fusarium solani pisi cutinase fusion proteins. J Biotechnol 100(1):55–64

Fexby S, Bulow L (2004) Hydrophobic peptide tags as tools in bioseparation. Trends Biotechnol 22:511–516

Hassinen C, Kohler K, Veide A (1994) Polyethylene glycol-potassium phosphate aqueous two-phase systems. Insertion of short peptide units into a protein and its effects on partitioning. J Chromatogr A 668:121–128

Johansson G, Walter H (2000) Partitioning and concentrating biomaterials in aqueous phase systems. Int Rev Cytol 192:33–60

Kepka C, Collet E, Roos F et al (2005) Two-step recovery process for tryptophan tagged cutinase: interfacing aqueous two-phase extraction and hydrophobic interaction chromatography. J Chromatogr A 1075:33–41

Persson M, Bergstrand MG, Bulow L et al (1988) Enzyme purification by genetically attached polycysteine and polyphenylalanine affinity tails. Anal Biochem 172:330–337

Rodenbrock A, Selber K, Egmond MR et al (2000) Extraction of peptide tagged cutinase in detergent-based aqueous two-phase systems. Bioseparation 9:269–276

Sagt CM, Muller WH, Boonstra J et al (1998) Impaired secretion of a hydrophobic cutinase by *Saccharomyces cerevisiae* correlates with an increased association with immunoglobulin heavy-chain binding protein (BiP). Appl Environ Microbiol 64:316–324

Steffens MA, Fraga ES, Bogle IDL (2000) Synthesis of protein purification tags for optimal downstream processing. Comput Chem Eng 24:717–720

Terpe K (2003) Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. Appl Microbiol Biotechnol 60:523–533