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Extraction of endoglucanase I (Cel7B) fusion proteins from *Trichoderma reesei* culture filtrate in a poly(ethylene glycol)–phosphate aqueous two-phase system

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Abstract

Endoglucanases (EGI) (endo-1,4-β-D-glucan-4-glucanohydrolase, EC 3.2.1.4, Cel7B) of *Trichoderma reesei* are industrially important enzymes. Thus, there is a great need for development of a primary recovery method suitable for large-scale utilization. In this study we present a concept applicable for large-scale purification of an EGI fusion protein by one-step extraction in a poly(ethylene glycol) PEG–sodium/potassium phosphate aqueous two-phase system. EGI is a two-module enzyme composed of an N-terminal catalytic module and a C-terminal cellulose binding module (CBM) separated by a glycosylated linker region. Partitioning of six different EGI constructs, containing the C-terminal extensions $(WP)_2$, $(WP)_4$ or the amphiphilic protein hydrophobin I (HFB) of *T. reesei* instead of the CBM were studied to evaluate if any of the fusions could improve the partition coefficient sufficiently to be suitable for large-scale production. All constructs showed improved partitioning in comparison to full length EGI. The $(WP)_4$ extensions resulted in 26- to 60-fold improvement of partition coefficient. Consequently, a relative minor change in amino acid sequence on the two-module protein EGI improved the partition coefficient significantly in the PEG 4000–sodium/potassium phosphate system. The addition of HFBI to EGI clearly enhanced the partition coefficient ($K=1.2$) in comparison to full-length EGI ($K=0.035$). Partitioning of the construct with $(WP)_4$ fused to the catalytic module and a short sequence of the linker [$EGI_{core\text{-}P5}(WP)_4$] resulted in the highest partition coefficient ($K=54$) and a yield of 98% in the PEG phase. Gel electrophoresis showed that the construct with the $(WP)_4$ tag attached after a penta-proline linker could be purified from the other bulk proteins by only a single-step separation in the PEG 4000–sodium/potassium phosphate system. This is a major improvement in comparison with the previously studied model (ethylene oxide–propylene oxide)–dextran system. Hence, this construct will be suitable for further optimization of the extraction of the enzyme in a PEG 4000–sodium/potassium phosphate system from culture filtrate.

Keywords: *Trichoderma reesei*; Aqueous two-phase systems; Endoglucanases; Enzymes; Proteins; Poly(ethylene glycol); Sodium phosphate; Potassium phosphate; Phosphates

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1. Introduction

An aqueous two-phase system consists of a mixture of two structurally different polymers, e.g. poly(ethylene glycol) (PEG) and dextran, or a polymer e.g. PEG and a high concentration of salt e.g. phosphate. Above a certain concentration the phase forming components will separate into two phases [1]. Aqueous two-phase systems offer a mild method to separate biomaterial due to the high water content in both phases [2]. The advantage of aqueous two-phase extraction is that cell separation, product purification and concentration can be obtained in one single step. Additionally, aqueous two-phase extraction is easy to scale up and suitable for primary recovery in large-scale applications. For industrial large-scale enzyme extractions PEG–salt systems are mainly used [3].

By altering the surface properties of a protein, e.g. by addition of fusion tags through genetic engineering, the partitioning behavior can be changed. For proteins used in industrial applications as compared to pharmaceuticals such surface changes should not be critical as long as the desired function of the molecule is retained. However, if indeed a removal of the tag is needed an enzymatic or chemical cleavage site between the target protein and the fusion tag has to be introduced [4]. The addition of short tryptophan-rich peptide tags has previously been shown to enhance partitioning to the more hydrophobic phase in different systems both in [ethylene oxide–propylene oxide random copolymer (EO–PO)]–dextran and PEG–sodium phosphate systems [5,6]. Three different proteins have been utilized as target proteins, a small model protein based on the staphylococcal protein A (Z) [5,7,8], the lipolytic enzyme cutinase [9] and the cellulase endoglucanase I (EGI) [10,11]. Recently, a small (M_r 6000) amphiphilic protein, hydrophobin I (HFBI) [12], has been fused to endoglucanase I (EGI) [13]. The addition of HFBI was shown to strongly direct the partitioning of EGI towards the detergent-enriched phase in cloud-point extraction systems e.g. $C_{12-18}EO_5$–water and Triton X-114–water [14].

In the current study we present partitioning of the two-module cellulase EGI (endo-1,4-β-D-glucan-4-glucanohydrolase, EC 3.2.1.4, Cel7B) of *Trichoderma reesei* in a PEG–phosphate aqueous two-phase system [15]. Cellulases have been utilized in different commercial applications, e.g. in textile industry [16] and pulp and paper industry [17]. EGI consists of two functionally and structurally distinct modules, a catalytic core and a cellulose binding module (CBM). An O-glycosylated linker region joins the two modules together. Purification of single cellulases is cumbersome since all cellulases have similar characteristics.

The purpose of this study was to develop an extraction method for purification of EGI from cell broth and bulk proteins applicable for large-scale use. PEG–salt systems are commonly used for commercial purifications of proteins [3] and therefore the PEG 4000–sodium/potassium phosphate system was chosen. To try to increase the partitioning of EGI to the PEG-rich top phase, two different model tags were investigated, namely the tryptophan-rich peptide tag based on a WP-motif and HFBI [10,11,14]. EGI has previously been genetically engineered to contain short C-terminal extensions including tryptophan residues or the protein HFBI, and the fusion proteins have been successfully expressed in *T. reesei* [10,11,13]. The effect of the localization of peptide tags and tag efficiencies have been studied in the model system (ethylene oxide–propylene oxide)–dextran system [10,11], which is a system particularly suitable for calculation of the tag effects to prove presence and estimate exposure of the tag. However, the (EO–PO)–dextran system is not suitable for large-scale utilization due to the high cost of the dextran polymer. Although the amphiphilic properties of HFBI have been shown to direct the EGI–HFBI fusion protein towards the detergent-enriched micelle phase in a CPE system some parameters having a negative impact on the large-scale use of CPE systems have been identified. The phase behavior has been shown to be sensitive to standard variations in fermentations, and phase separation difficulties have occurred due to low density differences between the phases and high viscosity of the micelle-enriched phase. Decreased binding capacity in the subsequent chromatography step used for further purification was also observed [18]. Therefore, in this work we have characterized the effect of the WP motif and HFBI as fusion tags

in an aqueous two-phase system applicable for commercial purification of the industrially important enzyme endoglucanase from culture filtrate.

2. Materials and methods

2.1. Cultivation and sample preparation

The fusion proteins with tryptophan tags were expressed under the *Aspergillus nidulans gpdA* promoter [19] in the *T. reesei* strain QM9414 cultivated on glucose-containing medium [20] as previously described [10,11]. Under these conditions endogenous production of cellulases is suppressed [21] and the only protein, which exhibits endoglucanase activity, is the fusion protein expressed under the *gpdA* promoter. This was performed to prevent interference of endogenous cellulase activity in the determination of partition coefficients of the different fusion proteins with the tryptophan tags. The recombinant strains with the constructs under the *gpdA* promoter (Fig. 1), described in Refs. [10,11], were cultivated according to previous publications [10,11] and the culture filtrate was further used in aqueous two-phase separations.

The VTT D-99703 strain producing the fusion protein EGI–HFB under the strong *cbh1* promoter was grown in a 15-l fermentor on minimal medium [20] supplemented with 2% lactose, 0.4% peptone and 0.1% yeast extract, which where used instead of glucose as carbon source. After 4 days of cultivation the mycelia were removed by filtration. EGI–HFB was purified by aqueous two-phase system adding 2% of $C_{12-18}EO_5$ (Agrimul NRE 1205, Henkel, Germany) to the culture filtrate containing EGI–HFBI fusion protein and other cellulases. Purification was performed to avoid background activity from other cellulases than the EGI–HFB fusion protein in the partitioning experiments in the PEG–salt system. The mixture was allowed to settle for 3 h at 27°C. After the phases were separated, an equal volume of isobutanol was added to the micellar phase to remove the surfactant. The fusion protein was further desalted on a BioGel P6 (Bio-Rad Laboratories, Hercules, CA, USA) column using 50 m*M* acetate buffer pH 5 and then used for partition studies.

2.2. Aqueous two-phase partitioning

PEG 4000 (molecular mass distribution of 3500–4500), NaH_2PO_4 and K_2HPO_4 were obtained from Merck, Darmstadt, Germany. Two-phase systems composed of 10% (w/w) PEG 4000 and 10% (w/w) sodium/potassium phosphate were prepared by weighing appropriate amounts of solid PEG and 40% (w/w) stock solution of sodium/potassium phosphate, with base/acid molar ratio of 15, and protein sample to a total mass of 10 g. The culture filtrate samples from *T. reesei* cultivations of the different recombinant strains gave fusion protein concentrations in the systems between 0.01 and 0.1 mg/ml. The concentration of EGI–HFBI sample in the system was 0.09 mg/ml. In the latter case minimal medium was added to obtain the final mass. The volume ratio V_{top}/V_{bottom} in the system was 0.47. The systems were mixed for about 30 min, placed in a water bath at 25°C for at least 10 min and briefly centrifuged at 2500 *g* to separate the phases.

The partitioning of the fusion proteins are described by the partition coefficient *K*, which is defined as $K = C_T/C_B$ where C_T and C_B are the concentrations of the substance in the top phase and bottom phase, respectively. All partition coefficients are average values from at least duplicate experiments from at least two different cultivations. The mass balance of EGI in the PEG–salt system was determined by measuring the total endoglucanase activity added to the system and the activity found in the different phases. The yields in top and bottom phase were calculated as the ratio between total EGI activity in the phase and the total EGI activity added to the system.

2.3. EG assay and peptide assay

The enzymatic activities in the phases were determined according to a standard assay for endoglucanase activity with the substrate *p*-nitrophenyl-β-D-cellobioside (pNPC) from Sigma (St. Louis, MO, USA) in 200 m*M* sodium acetate, pH 5.0, according to Ref. [22]. Polymer–detergent composition was kept at a constant level in all measurements to correct for activation and deactivation of the enzyme from the polymers/detergents as described

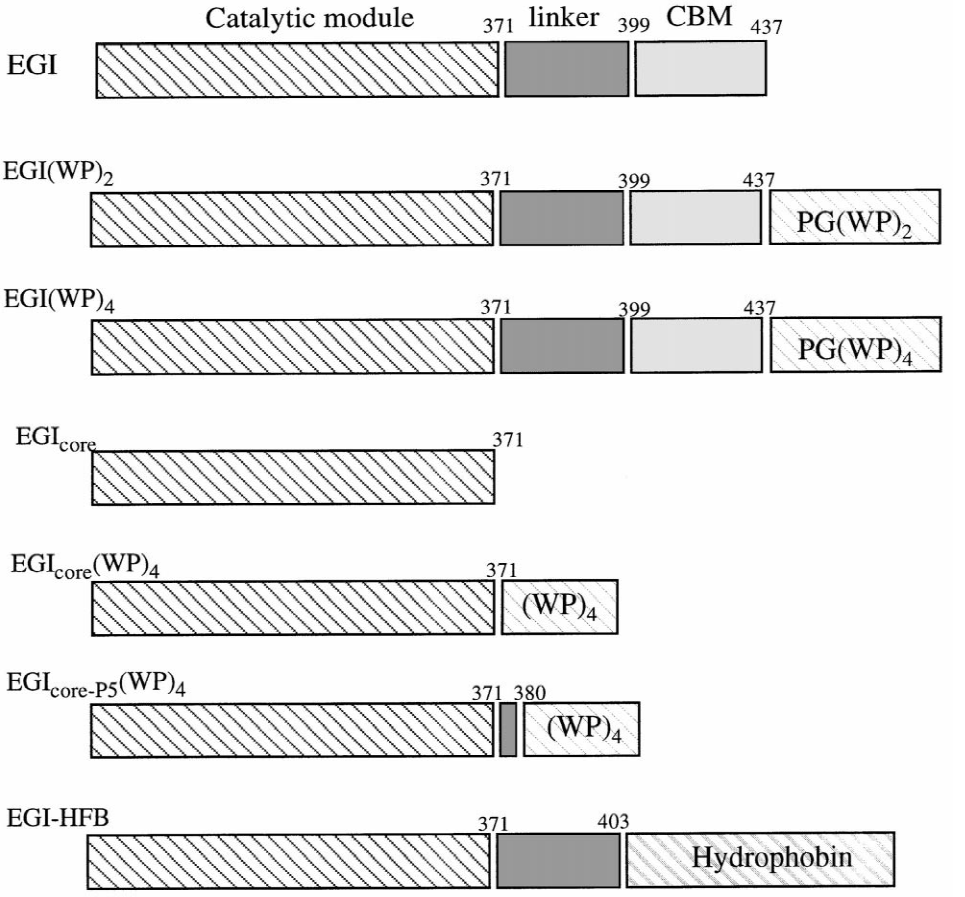

Fig. 1. Schematic picture of the different constructs: the full-length EGI (437 amino acid residues long [15]); EGI$(WP)_2$ is the EGI with the addition of P-G-$(WP)_2$ fused at the end of the CBM (443 residues long); EGI$(WP)_4$ is the EGI with the addition of P-G-$(WP)_4$ at the end of the CBM (447 residues long); EGI_{core} is the catalytic module of EGI (371 residues); $EGI_{core}(WP)_4$ is EGI_{core} with the addition of $(WP)_4$ (379 residues); $EGI_{core\text{-}P5}(WP)_4$ is EGI_{core} and a short stretch of the linker ending with five prolines with the addition of $(WP)_4$ (388 residues); EGI–HFB is hydrophobin I fused to the catalytic module and a part of the linker after residue 403. The catalytic module is between amino acid position 1 and 371 [28], the linker between 372 and 399 and the CBM between 400 and 437 [22].

in Refs. [10,11]. The absorbance contribution from the yellow color from the culture filtrate was withdrawn from the absorbance obtained from the activity assay since color distribution in the system was not even. The peptides, $(WP)_2$ and $(WP)_4$ (Synpep, Dublin, CA, USA) were partitioned in the same system as above, with glucose media as buffer according to Refs. [10,11].

2.4. Electrophoresis

To verify the partitioning of $EGI_{core\text{-}P5}(WP)_4$, reduced sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 4–12% gradient gel, Novex system (Novex, San Diego, CA, USA) was run. The gel was silver stained using Novex silver staining system and was scanned with a densitometer (Personal Densitometer SI, Molecular Dynamics, Sunnyvale, CA, USA).

3. Results and discussion

3.1. EGI and EGI_{core} partitioning

The different fusion proteins (Fig. 1) were par-

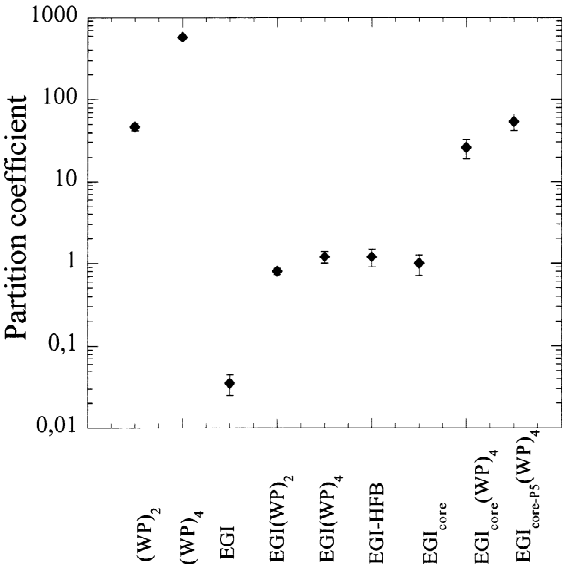

Fig. 2. Plot of the *K* values obtained from partitioning in the PEG 4000 (10%, w/w) and sodium/potassium phosphate (10%, w/w) system at 25°C. Partition coefficients of the peptides $(WP)_2$ and $(WP)_4$ and the proteins EGI, $EGI(WP)_2$, $EGI(WP)_4$, EGI–HFB, EGI_{core}, $EGI_{core}(WP)_4$ and $EGI_{core\text{-}P5}(WP)_4$.

titioned in the PEG–sodium/potassium phosphate system and the partitioning coefficients are presented in Fig. 2. The obtained yields are presented in Table 1. Full-length EGI showed a strong preference for the phosphate-rich bottom phase with a partition coefficient of 0.035, which indicates that other cellulases might partition to the bottom phase due to their similar properties. The deletion of the CBM and parts of the linker resulted in an increase in partition coefficient to 1.0. On the contrary in the polymer–polymer, (EO–PO)–dextran system a decrease in the partition coefficient was obtained when the CBM and parts of the linker were deleted [10,11]. Previously, exposure studies of the three-dimensional structure of the CBM [10,23] by the software GRASP [24] have shown that one tryptophan and three tyrosine residues are exposed on the surface. Theoretically, absence of these exposed aromatic amino acid residues would lead to a decrease of partition coefficient as was observed in the (EO–PO)–dextran system [10,11]. However, the factors, which affect the partitioning, are more complex in the PEG–salt system than the polymer–polymer, (EO–PO)–dextran system. One plausible explanation for the increase in partition coefficient might be the absence of the complete heavily glycosylated and charged linker region. The linker region presumably directs the protein more strongly to the hydrophilic salt-rich phase in the PEG–salt system than to the dextran-rich phase in the (EO–PO)–dextran system used in previous studies. Additionally, in a polymer–salt system the entropic effect is larger than in a polymer–polymer system [25]. Due to the size difference between the full-length EGI and EGI_{core} one would expect the larger EGI to be excluded from the PEG 4000 phase to a greater extent than EGI_{core}. Consequently, this might be another explanation for the observed increase in partition coefficient.

Table 1
The yields in the top PEG phase and bottom salt phase for EGI, EGI_{core} and the fusion proteins $EGI(WP)_2$, $EGI(WP)_4$, EGI–HFB, $EGI_{core}(WP)_4$ and $EGI_{core\text{-}P5}(WP)_4$ in 10% (w/w) PEG and 10% (w/w) phosphate system at 25°C are presented

Construct	Yield (%) Top phase	Yield (%) Bottom phase
EGI	1	96
$EGI(WP)_2$	33	78
$EGI(WP)_4$	41	48
EGI–HFB	30	78
EGI_{core}	40	70
$EGI_{core}(WP)_4$	90	2
$EGI_{core\text{-}P5}(WP)_4$	98	<1

3.2. EGI–HFB fusion protein partitioning

Here for the first time the amphiphilic HFBI from *T. reesei* has been utilized as a fusion tag to direct the target protein to the PEG-rich phase in a PEG–salt system. This tag has recently been shown to have a strong preference for the detergent-enriched phase in several cloud-point extraction systems [14]. HFBI contains several aliphatic amino acid residues but few aromatic residues [12]. It has previously been seen that the aliphatic amino acid residue isoleucine, added to the model protein ZZ, increased the partition to the PEG-rich phase in the PEG–potassium phosphate system. However, the aliphatic residue was not as effective as the aromatic and polar tryptophan residue [8,26]. Consequently, the addition of HFBI to EGI clearly enhanced the partition coefficient from 0.035 to 1.2 and a yield of 30% was obtained in the PEG-rich phase (Fig. 2 and Table 1).

To optimize the partitioning, several other systems with different concentration of PEG and salt and different molecular masses of PEG (PEG 1500 and PEG 4000) were tested (data not shown). However, no improvement was obtained compared to the 10% (w/w) PEG 4000–10% (w/w) sodium/potassium phosphate system. The obtained increase in partition coefficient and yield was not sufficient for utilization of this construct in large-scale applications in the PEG–sodium/potassium phosphate system. However, a construct without the linker part with HFBI directly fused to the C-terminus of EGI_{core} could be useful.

3.3. Partitioning of EGI fused with tryptophan tags

It has previously been shown that proteins tagged with short peptides containing tryptophan residues have an enhanced partitioning to the PEG-rich phase in PEG–salt systems [5,7–9]. However, this has been demonstrated with only small one-module proteins. Consequently, investigations of how short peptide tags affect the partition of larger proteins, as in this case EGI, a glycosylated two-module enzyme is an important test of the tag concept in PEG–salt systems.

The addition of the peptide $(WP)_2$ to the full length EGI increased the partitioning to the PEG-rich phase (Fig. 2). The fusion tag was designed to give optimal solvent exposure of the tryptophans by avoiding secondary structure conformation [10,11]. This was performed by addition of proline residues. Proline residues have previously been shown to partition close to unity [26] and therefore they should not counteract too much the primary goal of the tag, which is to enhance partitioning to the hydrophobic PEG-rich phase. Furthermore, by raising the tryptophan content of the peptide tag the target protein showed enhanced partitioning to the PEG-rich phase, as for the peptides (Fig. 2). Thus, the addition of the $(WP)_4$ tag to EGI further improved the partition coefficient of the fusion protein in comparison to the $(WP)_2$ tag added to EGI (Fig. 2). Consequently, $EGI(WP)_4$ resulted in a better partition improvement than $EGI(WP)_2$ (Table 2). The partition improvement (PI) is defined as [9]:

Table 2
The PI (partition improvement) and TEF (tag efficiency factor) for EGI and the fusion proteins $EGI(WP)_2$, $EGI(WP)_4$, $EGI_{core}(WP)_4$ and $EGI_{core\text{-}P5}(WP)_4$ in 10% (w/w) PEG and 10% (w/w) phosphate system at 25°C are presented. PI and TEF are calculated according to Eqs. (1) and (2), respectively

	Partition improvement	Tag efficiency factor
$EGI(WP)_2$	23	0.81
$EGI(WP)_4$	34	0.56
$EGI_{core}(WP)_4$	26	0.51
$EGI_{core\text{-}P5}(WP)_4$	60	0.63

$$\mathrm{PI} = \frac{K_{\text{tagged protein}}}{K_{\text{protein}}} \quad (1)$$

For the truncated forms of EGI, $EGI_{core}(WP)_4$ and $EGI_{core\text{-}P5}(WP)_4$, the partition coefficients were higher than for $EGI(WP)_4$ partly due to the higher partition coefficient of the reference protein EGI_{core} (Fig. 2). $EGI_{core\text{-}P5}(WP)_4$ partitioned almost exclusively to the PEG-rich phase and resulted in a yield of 98% (Table 1) of the tagged protein in the top phase based on activity measurements. Other cellulases are assumed to partition to the salt phase since the full-length EGI partitioned exclusively to this phase. Purification of $EGI_{core\text{-}P5}(WP)_4$ was confirmed by estimation of the yield with a silver stained SDS–PAGE gel (Fig. 3). Thus, purification of $EGI_{core\text{-}P5}(WP)_4$ from culture filtrate was obtained in only one extraction step since the bulk of proteins mainly partitioned to the salt-rich phase, which is a major step forward.

3.4. Tag efficiency

The tag efficiency factor TEF is defined as [5]:

$$\mathrm{TEF} = \frac{\log K_{\text{tagged protein}} - \log K_{\text{protein}}}{\log K_{\text{tag}}} \quad (2)$$

A combination of Eq. (1) with Eq. (2) results in:

$$\log \mathrm{PI} = \mathrm{TEF} \times \log K_{\text{tag}} \quad (3)$$

The addition of the peptide $(WP)_2$ to EGI resulted in a better tag efficiency than addition of $(WP)_4$ (Table 2). The data obtained in the current study correspond to previously presented data in the PEG–

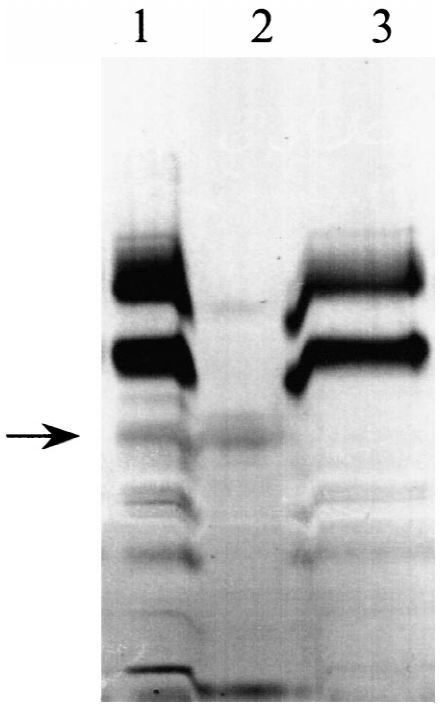

Fig. 3. SDS–PAGE analysis of the partitioning of $EGI_{core\text{-}P5}(WP)_4$. The samples have been volume adjusted so that a direct visual comparison can be made between the lanes. Hence, if 100% of the target protein is recovered, the band of the sample should have the same intensity as in the starting material. The arrow indicates the position of $EGI_{core\text{-}P5}(WP)_4$. Lanes 1, the culture filtrate from $EGI_{core\text{-}P5}(WP)_4$ cultivation; 2, the proteins in the PEG phase; 3, the proteins in the salt phase.

salt system from fusions of cutinase [9] and the model protein ZZ [6] where the TEF for fusions with two and four additional tryptophan residues were about 0.8 and 0.6, respectively. Furthermore, slight differences in $(WP)_4$ tag efficiency were observed for the different EGI variants. However, in accordance with recent studies in the (EO–PO)–dextran system [11] the TEF was higher when the $(WP)_4$-tag was attached to the catalytic module of EGI with a short stretch of the linker than when the tag was attached to the CBM or directly to the catalytic module. The difference in TEF might be related to differences in exposure of the tag. The fusion protein $EGI_{core\text{-}P5}(WP)_4$ has a short stretch of the linker region of EGI, which ends by five proline residues, as a spacer. Polyproline could form a special form of an extended helical structure [27], which might be one reason why this spacer appears to be suitable to utilize for better exposure of the tag. Hence, the $EGI(WP)_4$ construct, which resulted in a lower TEF value, only has a short spacer of Pro–Gly added before the fusion peptide and for the $EGI_{core}(WP)_4$ construct, which lacks spacer, the lowest TEF was obtained. Although the tag efficiencies are in the same range in both the PEG–salt system and previously studied (EO–PO)–dextran system [10,11] the partition improvement is larger in the PEG–salt system. This is due to the higher partition coefficient of the tag in the former system.

4. Conclusions

We have demonstrated that the relatively moderate change of adding $(WP)_4$ as well as the protein HFBI to a M_r 62,000 large glycosylated two-module protein strongly affects the partition properties in a PEG 4000–sodium/potassium phosphate aqueous two-phase system. Furthermore, the fusion of $(WP)_4$ to the EGI catalytic module after a penta-proline linker [$EGI_{core\text{-}P5}(WP)_4$] resulted in a partition coefficient of 54, a yield of 98% and an almost complete purification by a single step extraction in the PEG 4000–sodium/potassium phosphate system. Hence, the concept of utilizing the fusion protein $EGI_{core\text{-}P5}(WP)_4$ in a PEG 4000–sodium/potassium phosphate system should provide the basis for further optimization of a large-scale extraction process for the EGI catalytic module from culture filtrate.

Acknowledgements

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