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Production of Cysteine Proteases by Recombinant Microorganisms: A Critical Review

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Abstract:*Cysteine Proteases are groups of enzymes which able to degrade protein. These enzymes are commonly found in nature in many plant families and rich in known fruits such as in papaya, pineapple, and kiwi. However, the highest enzyme concentration is usually obtained from unripe fruits. In addition to their traditional uses of cysteine proteases as meat tenderizers, they have nowadays wider applications in the field of food, pharma, and textile industries. Beside the traditional extraction of these enzymes from tropical fruits, especially from papaya fruit, this enzyme was successfully expressed in many microbial cells for industrial production of this important group of enzymes. This review is focused on providing more updated information about the recent development in the production of cysteine proteases using recombinant microorganisms belong to different classes of biofactories.*

Keywords: *Enzyme production,Cysteine proteases, Recombinant microorganisms, Papain, Pro-papain*

I. Introduction

Enzymes are important biocatalysis and play important role nowadays in biorefinery, textile, wood, detergent, feed, food, pharmaceutical, and medical industries. These include different enzyme groups such as amylases, proteases, lipases, acylases, oxidases, transferases, and many others [1-8]. Therefore, they are considered as main components of the growth of wellness industries in general [9]. However, amylases, proteases, and lipases contribute in the large fraction of the enzyme market volume. Proteases are classified based on their catalytic activities into five main classes: metallo, aspartic, cysteine, serine, and threonine [10]. Some enzymes belong to proteases groups characterized by their high tolerance of extreme environment in terms of temperature and pH [11,12]. Beside the fact that, enzymes exist in almost all living organisms, industrial production of the enzymes is mainly carried out using microbial cells which provide high yield in shorter time compared to other higher organisms. However, for enzymes produced mainly in higher organisms such as plant, insect, and mammalian cells, many attempts have been done to construct the genes responsible for enzyme production and successfully expressed in different organisms. In particular, cysteins proteases are exist in different types of tropical fruits and mainly produced by papaya fruit and leaves *Carica papaya* in industrial scale. Our recent review covered the production, extraction, purification, and diverse application of this enzyme [13]. In recent years, many successful attempts were done to express different types of cysteine proteases in microorganisms. This review highlighted the recent attempts done for gene expression and production of cysteine protease in different types of eukaryotic and prokaryotic cells.

1. Cysteine Papain of Recombinant Microorganisms

Due to the versatility of papain and its high degree of applicability in different industrial sectors, it is anticipated that the enzyme be obtained in considerable quantities thus allowing for ample supply for both research and commercial activities. However, it should be noted that papain enzyme extraction and preparation from plant source would be greatly influenced by the climatic conditions for the growth of said plants as well as the methods applied in the extraction and subsequent purification processes. Thus, plant extracted papain would be unsuitable in applications requiring highly pure homogenous preparation of the enzyme. Therefore, in order to solve this particular issue, it is beneficial to acquire a wild-type recombinant form of the enzyme which would subsequently allow for introducing selective modifications into the primary sequence thus tailoring the protein properties through directed evolutions or rational design choices [14]. Hence, it could be established that the main objective of production and recovery of recombinant papain is to ultimately produce a stable papain-like cysteine protease through structure based site-directed mutagenesis as demonstrated by Thakurta*et al.* [15]. Henceforth, it is of great import that efficient protein expression systems be available for obtaining further

insights into this crucial protease family. In all, this review would attempt to summarise the heterologous expression methods that have been documented in expressing papain-like proteases, the ensuing activation to active proteins followed by the associated purification and storage parameters required. Finally, a discussion would also be included on modification made on existing expression methods allowing for increase in yield and productivity of papain-like protease.

2. Expression of methods

For this review, four different expression of recombinant papain-like cysteine protease will be discussed. These methods include the use of bacteria, yeast, baculovirus (insect cells) and mammalian cells as the expression vectors. Table 1 below shows the propapain yield and the important experimental remarks followed by Table 2 highlighting the overall advantages and disadvantages of the respective methods outlined previously. Table 3 then shows the different vectors, the papain-like protease generated, the host strain, the purification methods used and the yield for the different combinations as compiled and summarised byBrommee*t al*. [16].

Table 1: Propapain yield and important experimental findings with respect to the different expression vectors in pioneering experiments

Expression Vectors	Pro-papain Yield (mg L^{-1} of culture)	Experimental Remarks	References
Bacteria *Escherichia coli*	~3	- Expression inhibited by the 26 amino acid signal peptide - Recombinant propapain in the form of insoluble granular aggregates	[17] [18]
Baculovirus/ insect *Autographacalifornica/ Spodopterafrugiperda*	~0.3	- Baculovirus expression system is not ideal for mutagenesis-based structure-function analysis of propapain	[19,20]
Yeast *Saccharomyces cerevisiae*	~1.7	- Propapain expressed in soluble form within the yeast - Intermediate yield	[21,22]

Table 2: Summary on the advantages and disadvantages associated with the expression of papain-like cysteine protease through different vectors

Expression Vector	Advantages	Disadvantages/ Concerns
Escherichia coli	- Ease of manipulation of the expression vector - Rapid growth of *E. coli* - Require inexpensive media	- Ensuring high transcription and translation rates - Controlling toxicity of heterologous gene product - Maintaining protease solubility - Preventing proteolytic degradation of the expressed protein
Yeast	- Allow post-translational modifications i.e. glycosylation - No expression and secretion of endogenous cysteine endoproteases - Minimal secretion of endogenous protein (*Pichia* system) - Simple purification procedure	- Potential for high mannose glycosylation associated with certain yeast species
Baculovirus	- Allow post-translational modifications	- Expression of several endogenous cathepsin-like activities that interfere in recombinant protease analysis - Purification procedure is critical with low error threshold - Lower yield in the majority of cysteine protease species
Mammalian cell	- High degree of flexibility - Used almost exclusively for research purposes	- High costs associated with mammalian cell cultures - Expression of numerous endogenous cathepsins requires complex purification procedure.

Table 3: Summary of different experimental methods in papain-like cysteine protease expression using different vectors [16]

Host species	Protease	Host Strain/Expression vector or construct	Purification	Yield (mg L^{-1})
E. coli	Cathepsin B (human)	BL21(DE3)pLyS/pET3A	Sepharose S	3.0
	Cathepsin B (*L. Mexicana*)	M15pREP4/pGL180(CPB2.8δCTE)	Ni^{2+} - NTA-agarose or Mono Q, Sephadex G50	>3.5
	Cathepsin K (human)	LW29(DE3)/pET16b JM109/pQE-30	Superdex 75 Ni^{2+} - NTA-sepharose	50 54
	Cathepsin L (human)	BL21(DE3)/pCtsL BL21(DE3)pLysS/pET811	Sephadex G-75 Sephacryl S-200	1.3
	Cruzain (*T. cruzi*)	DH5α/pCheYTc	DEAE-sepharose	20-30
	Cathepsin S (human)	BL21(DE3)pLysS/pKG2-1 K12(λDE3)/pT73.3	Sephacryl S-200 Thiopropyl-Sepharose 6B	2 14
	Cathepsin C (bovine)	BL21/p8PM	ND	0 (deg)
	Caricain (*C. papaya*)	BL21(DE3)/pET3aPpΩI	ND	ND
	Falcipain-2 (*P. falciparum*)	AD(DE3)pLysS/pRSET M159pREP4/pQE	Ni^{2+} - NTA, Q-Sepharose	ND
Yeast	Cathepsin C (bovine)	*C. boidinii* SK741/pFCTC-S1	ND	12
	Cathepsin B (*L. major*)	*P.pastoris* GS115/pPIC9	ND	33
	Cathepsin B2 (*S. mansoni*)	*p. pastoris* X33/pPICZα	PD10, Mono S	40-60
	Cathepsin F(human)	*P. Pastoris* GS115/pPIC9	Source 15S, Superdex-75 HiTrap SP	0.17 1.0
	Cathepsin K (human)	*P. Pastoris* GS115/pPIK9K	*n*-Butyl-Sepharose	38
	Cathepsin L (human)	*P. Pastoris* GS115/pPPIC9	HPLC on vydae C4	10
	Cathepsin L (*C. sinensis*)	*P. Pastoris* X33/pPICZα	Ni^{2+} - NTA agarose, Sephacryl S-200	19.7
	Cathepsin X (human)	*P. Pastoris* GS115/pPIC9	CM-Sephadex	5
	Cathepsin L (human)	*S. cerevisiae* BJ3501/YpDC222	S-Sepharose	0.0025
	Cruzipain-2 (*T. cruzi)*	*S. cerevisiae* BJ3501/YpDC280	Thiopropyl-Sepharose 6B, con A-Sepharose	0.029
	Cathepsin S (human)	*S. cerevisiae* BJ3501/YpDC222	Thiopropyl-Sepharose 6B	0.17
	Papain (*C. papaya*)	*S. cerevisiae* BJ3501/YpDC222	Thiopropyl-Sepharose 6B	ND
Baculo-virus	Cathepsin B (human)	S*f*9/pFastBac1(Bacmid)	SP-Sepharose	>25
	Cathepsin B1 (*S. mansoni*)	S*f*9/ND	ND	ND
	Cathepsin C (human)	High Five/pVL1393	*n*-Butyl-Sepharose FF, Q-Sepharose FF	10-15, 20-30
	Cathepsin C (rat)	High Five/pVL1393	Ni^{2+} - NTA, butyl-Sepharose FF, Q-Sepharose FF	50
	Cathepsin C (*S. japonicum*)	High Five/pMHis+BacPAK6 (Bacmid)	Ni^{2+} - NTA	
	Cathepsin K (*M. fascicularis*; monkey)	S*f*21/vBacMncatK	S-Sepharose	7.5
	Cruzipain (*T. cruzi*)	S*f*21/pFastBacHTb	Ni^{2+} - NTA or Con A-Sepharose, Mono Q, Mono P	2
	Cathepsin S (mouse)	S*f*9/pFastBac1 (Bacmid)	Ni^{2+} - NTA or HiPrepQXL	>1
	Cathepsin S (rat)	S*f*9/pFastBac1(Bacmid)	Ni^{2+} - NTA or HiPrepQXL	>1
	Cathepsin S (humanized rod enz)	S*f*9/pFastBac1 (Bacmid)	Ni^{2+} - NTA or HiprepQXL	>1
Mammalian Cells	Cathepsin K (*Rhesus* monkey)	CHO/pcDNA3.1	Unpirified cell extract	ND
	Cathepsin B (human)	HeLa/vTF7EMCV	Cathepsin B mabAffi-gel 10	$0.2/10^8$ cells
	Cathepsin L (human)	A431 epidermal carcinoma cell line/pHa-MDR1+pHaMEPh	Immune precipitation, protein A-Sepharose or Con A-Sepharose	15 x basal AU/10^5 cells
	Cathepsin L (mouse)	NIH3T3 or KNIH/pHaMDR1+pcosMEP5A	Protein A-Sepharose or Con A-Sepharose	3 x basal AU/10^6 cells

3. Expression Process Modification

In the expression process of papain-like cysteine proteases, it is apparent that the yield of the pro-papain is relatively low thus making most of expression methods uneconomical and unsuitable for large scale applications. Hence, modifications are required in order to improve the overall yield and the feasibility of producing recombinant papain-like cysteine proteases. In regards to the expression using *E. coli*,*Choudhury et al.* [23] proposed modification to the expression through the use of two T7 promoter based vectors namely pET28a+ and pET30 Ek/LIC. Here, they introduced an alternative refolding protocol scheme resulting in a yield of around 400 mg of properly folded pro-papain per litre of *E. coli* culture which is a significant increase on the amount produced in earlier experiments. It should be mentioned that the pro-papain is expressed in the form of insoluble granular aggregates (inclusion bodies). Nonetheless, it was found that autocatalytic processing which will be discussed in greater detail within the next section is applicable in the conversion of the refolded pro-papain to active mature papain.

4. Precursor Activation

Prior to delving into the process of activating the precursor, it should be mentioned that papain-like cysteine proteases comprise of a pro-region which fulfil three varied functions [16]. These functions include acting as the overseeing domain for the transport to the endosomal/ lysosomal compartment as well as ensuring that the correct folding process of the catalytically mature protein is carried out. Additionally, the pro-region is also responsible in suppressing the activity of the proenzyme.In structure, the length of the pro-region differs considerably comprising of peptide length between 38 amino acids found for cathepsin X to 251 amino acids for cathepsin F. The majority of the heterologous expression systems for cysteine protease as discussed in this report result in the production of proteotically inactive proenzymes that are relatively stable with slightly acidic to slightly alkaline pH. As such, the removal of the pro-region which suppress the proenzyme is a must to allow for the generation of proteotically active proteases.

The means in which this pro-region is removed usually involve autocatalytic activation through the incubation of the precursor proteins in acetate buffer of pH around 4 to 4.5. Actual autocatalytic activity has been shown to be facilitated through the presence of negatively charged glycosaminoglycan such as the normally utilised dextran sulfate [24]. It should however be noted that exopeptidases namely cathepsins X and C were shown to be unaffected and thus required additional protease before achieving full activation [25]. This processing of procathepsins has been shown to undergo dramatic enhancement upon the addition of porcine pepsin (0.6 mg mL-1) into the buffer. Bromme*et al.* [16] reported the specific parameters that should be attained before addition of the pepsin which includes adjustment of the pH to 4 and incubation temperature set at 37 °C. The activation process could be halted through increasing the pH to 5.5 hence allowing greater control to the researcher on the overall process.

5. Recombinant Papain-like Cysteine Protease Purification

The addition of porcine pepsin in the activation process as discussed in the previous section enables simpler purification process. This is due to the secretion of protease zymogens by the pepsin which in turn hydrolyses most of the proteins present within the culture medium. As mentioned, this proteolytic clearance not only simplifies subsequent purification steps but more importantly, it also permits the obtainment of electrophoretically homogeneous protein after one chromatography step [16]. Bromme*et al.* [16] also reported that the combination of the chromatographic resins should be selected based on the physicochemical parameters of the papain-like cysteine protease produced in the particular expression step. The chromatography steps used by various publications on the purification process for different papain-like cysteine proteases are shown in the previous Table 3

6. Storage of Recombinant Papain-like Cysteine Proteases

Normally, cysteine proteases are known to be unstable enzymes with high tendencies towards undergoing autocatalytic degradation, oxidation as well as protein denaturation [16]. Exposing let alone storing the enzymes at room temperature with sub-molecular concentration will ensure rapid loss of enzymatic activity. Bromme, *et al.* [16] observed that some of the cathepsins i.e. cathepsin K are unstable at -20 °C and required storage at -80°C. Here, they established that the micromolar stocks of cathepsins L, K, V, S and F were able to retain full activity for years upon storage in a pH 5.5 acetate buffer containing 1 mMdithiothreitol and 1 mM EDTA at -80°C. Care should also be given in the storage of the protease stock solutions with recommendation for freezing in small aliquots to avoid frequent melting and freezing thus resulting in considerable loss of enzymatic activity. In regards to cathepsins B, L and H, it was shown that they are stable in the form of reversible mercury complexes [26]. Through these reversible mercury complexes, the cathepsins were shown to retain their stability at room temperature with the reactivation process requiring just the addition of 10 mM EDTA. Nevertheless, this process is not applicable to all cathepsins and other papain-like cysteine proteases.

Finally, there are also suggestions that papain-like cysteine proteases could be reversibly inactivated through the addition of methyl thiosulfanate (MMTS) in the molar ratio of the inhibitor to the enzyme of 1.25 to 1 (1.25:1) [27].

II. Conclusion and Future Perspective

Throughout this short review, it is also observed that there are numerous protein expression systems that could be carried out in the generation of papain-like cysteine proteases utilizing different types of microbial biofactories which allowing for minimal reliance on using edible fruits as sole source of this group of enzymes. With the variations of the cysteine proteases, the best purification step and storage methods should then be considered due to their different natures. It could also be seen that each of the expression system possesses specific upsides and downsides in which the researchers would have to decide on which systems would be most appropriate for their applications. Further improvement of the expression level in cell cellular level and development of cultivation strategy of the recombinant cells for high yield production of cysteine protease in large scale is needed to decrease the production cost of this industrially important enzyme.

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