African Journal of Biotechnology Vol. 10(81), pp. 18829-18832, 16 December, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2761
ISSN 1684–5315 ©2011 Academic Journals

Full Length Research Paper

Recovery of recombinant bromelain from *Escherichia coli* BL21-AI

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Accepted 7 November, 2011

In spite of the fact that commercial bromelain supplements are available in the market, to date, none of them are produced and formulated from recombinant forms. They are extracted and purified (often partially) from the stem and fruit of pineapple (*Ananas comosus*). This makes the production of bromelain very difficult, less reliable, often contaminated and expensive. In this study, a recombinant bromelain was expressed as soluble (active) and insoluble (inactive) enzyme in *Escherichia coli* BL21-A1. The enzyme fractions were purified using Ni-NTA His•Bind resin with the aid of an automated fast protein liquid chromatography (FPLC) system. Purification fold and percentage yield of the purified active bromelain were found to be 3.7 and 64%, respectively. SDS-PAGE results showed that the enzyme was purified to near homogeneity and the soluble form exhibited a single band with molecular weight of about 45 kDa.

Key words: Bromelain, Ni-NTA His•Bind resin, fast protein liquid chromatography (FPLC), *Ananas comosus*.

INTRODUCTION

Plant tissues, animal organs and microorganisms are the most important sources of proteins and enzymes. For instance, enzymes from plants and animals, mostly proteases, are still in the markets and some of them are of commercial relevance. Proteases are enzymes that catalyse the hydrolysis of peptide linkages in proteins. Industrially, proteases are the most important type of enzymes as they constitute about 60% of all commercial enzymes in the world (Lucia and Tomas, 2010). They are largely used in food, pharmaceutical and detergent industries.

Microbial enzymes have been substituting those obtained from other sources and might now account for almost 90% of the total market (Illanes, 2008). This is due to the fact that microbial cells are excellent systems for enzyme production. In fact, they are metabolically vigorous, very versatile, easy to propagate, simple to manipulate, possess simple nutritional requirements and their supply is independent of season. These factors have stimulated extensive research works on recombinant proteins.

The use of purified proteins for therapeutic purposes has been in existence for many decades. Hormones such as insulin and human growth hormones, and other proteins were extracted and purified from blood and other tissues before the emergence of recombinant DNA technology (Paul, 2004). Enzymes constitute the largest portion of these purified proteins for industrial and therapeutic applications. Since the approval of insulin in 1982, hundreds of recombinant drugs including enzymes have been approved and become available as extremely valuable therapeutic options (Theo, 2008).

The name 'bromelain' was originally given to a mixture of proteases found in the juice of the stem and fruit of pineapple (*Ananas comosus*). Even now, bromelain is still used as collective name for the enzymes found in various members of *Bromeliaceae* family. The major peptidase present in the extracts of plant stem is called 'stem bromelain' while the major enzyme fraction found in the juice of pineapple fruit is called 'fruit bromelain'. Some other minor cysteine endopeptidases (*ananain, comosain*) are also present in the pineapple stem bromelain.

Stem bromelain (E.C. 3.4.22.32), belonging to papain family, is a glycosylated single chain protein containing of 212 amino acid residues including seven cysteines, one

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of which is involved in catalysis (Bitange et al., 2008). Pure stem bromelain is stable when stored at –20°C and has optimum pH activity at 6 to 8.5. The optimum temperature range for the enzyme is 50 to 60°C. It is mostly activated by cysteine while hydrogen sulphide and sodium cyanide are less effective (Maurer, 2001). However, heavy metals (like mercury and silver) and E–64 deactivate the enzyme.

Bromelain also has great therapeutic applications. It was firstly introduced as a therapeutic compound in 1957 (Gregory and Kelly, 1996). Bromelain actions include: (i) inhibition of platelet aggregation; (ii) fibrinolytic activity; (iii) anti-inflammatory action; (iv) anti-tumour action; (v) modulation of cytokines and immunity; (vi) skin debridement properties; (vii) enhanced absorption of other drugs; (viii) mucolytic properties; (ix) digestive assistance; (x) enhanced wound healing; and (xi) cardiovascular circulatory improvement (Gregory and Kelly, 1996). It is also used in food processing and baking industry (Lyons, 1982), meat tenderization and as a dietary supplement (Ravindra et al., 2008).

The use of conventional methods renders the production and formulation of bromelain very challenging, less reliable, often contaminated and expensive. Moreover, downstream processing is one of the major cost factors (up to 50%) in bioprocess. Hence, it is vital to develop efficient and cost effective processes that contribute towards the development of highly purified and cheaper bromelain. This study was geared towards the recovery of recombinant bromelain that were over expressed in *E. coli* BL21-A1.

MATERIALS AND METHODS

Luria Bertani (LB) growth media used was a product of Merck, Germany. L–Cysteine, L–arabinose and N-α-Cbz-L-Gln-*p*-nitrophenyl ester were purchased from Sigma Chemicals (USA). All other chemicals used were pure and of analytical grade.

BL21-A1 *Escherichia coli* strain was obtained from Invitrogen (USA) and used throughout the study.

Over expression of recombinant bromelain

E. coli cells harboring recombinant bromelain gene were grown over night in Luria Bertani (LB) media, containing 100 µg/ml ampicillin (Amid et al., 2011). The overnight culture was then diluted 100 fold in a fresh LB media containing 100 µg/ml ampicillin and was grown at 37°C and agitated at 250 rpm in shake flasks and then a 2-L bioreactor up to a cell density with OD_{600nm} = 0.4. Inducer (L-arabinose) was then added to reach a final concentration of 0.2% and the induction continued for 4 h. Cells were harvested from the spent media by centrifugation (8,000 rpm, 4°C and 20 min) and stored at –20°C until required for use (Ismail and Amid, 2008a and b).

Recovery of recombinant bromelain

The harvested cells were subjected to sonication (sonicator, 150v/t model, Biologics, Inc. USA) on ice using 6 to 10 s burst with 10 s interval at high amplitude. This was followed by centrifugation at 12000 rpm and 4°C for 30 min and the supernatant was collected and purified by AKTA purifier FPLC system (GE Healthcare Bio-Sciences, USA). The enzymes were purified under native and denaturing conditions according to the manufacturer's instructions. A glass column for chromatography (4.6 × 100 mm, Life Technologies, California) was filled with 1 ml of Ni-NTA His•Bind resin (Novagen, Germany). The FPLC system was set at flow rate of 1 ml/min.

Protein determination

The concentration of proteins present in the enzyme preparations were determined by Bradford's (1976) method using bovine serum albumin as standard.

SDS-PAGE

After each step of enzyme recovery and purification, the protein fractions were tested by SDS-PAGE in 12.5% polyacrylamide gels and visualization was performed by staining with Coomasie Brilliant blue R-250 (Laemmli, 1970).

Measurement of proteolytic activity

The assay for proteolytic activity was carried out by the Silverstein's (1974) method and modified to reach optimal conditions of the enzyme. The activity was studied using N-α-Cbz-L-Gln-*p*-nitrophenyl ester as substrate. Assays were made at 37°C in 0.1 M Tris–HCl buffer (pH 8.0), containing 25 mM cysteine and 1 mM of the substrate in the reaction mixture. Liberation of *p*-nitrophenol was followed spectrophotometrically at 405 nm (Secomam-Anthelie 5, France). An arbitrary enzyme activity unit (Ucbz) was defined as the amount of protease that released one micromole of *p*-nitrophenolate per min in the assay conditions (Maria et al., 2008).

RESULTS AND DISCUSSION

Recombinant bromelain was successfully expressed in *E. coli* BL21-AI as native (soluble) and denatured (insoluble) enzyme as presented in Figure 1. The samples from each step of the purification technique were analyzed by SDS-PAGE as shown in Figure 1a and c for soluble and insoluble bromelain, respectively. It could be seen clearly from these figure that only few target proteins were eluted in unbinding fractions and the bromelain was purified to almost homogeneity in one affinity purification step. The molecular weight of the purified soluble enzyme (containing six-His tag) was found to be approximately 45 kDa on SDS–PAGE (Figure 1b). This value closely correlated with the size of the natural bromelain gene (D14059.1). Moreover, the value fell within the range of molecular masses of plant cysteine proteases (25 to 75 kDa) as reported by Dubey et al. (2007). Soluble recombinant proteins often have correctly folded three-dimensional conformation; hence, normally bioactive in nature (Hans and Kim, 2005). However, the insoluble protein has unfolded structural conformation. This explains why single band was obtained for soluble bromelain (Figure 1a and b), while multiple bands were seen in the insoluble form (Figure 1c). The various bonds stabilizing the

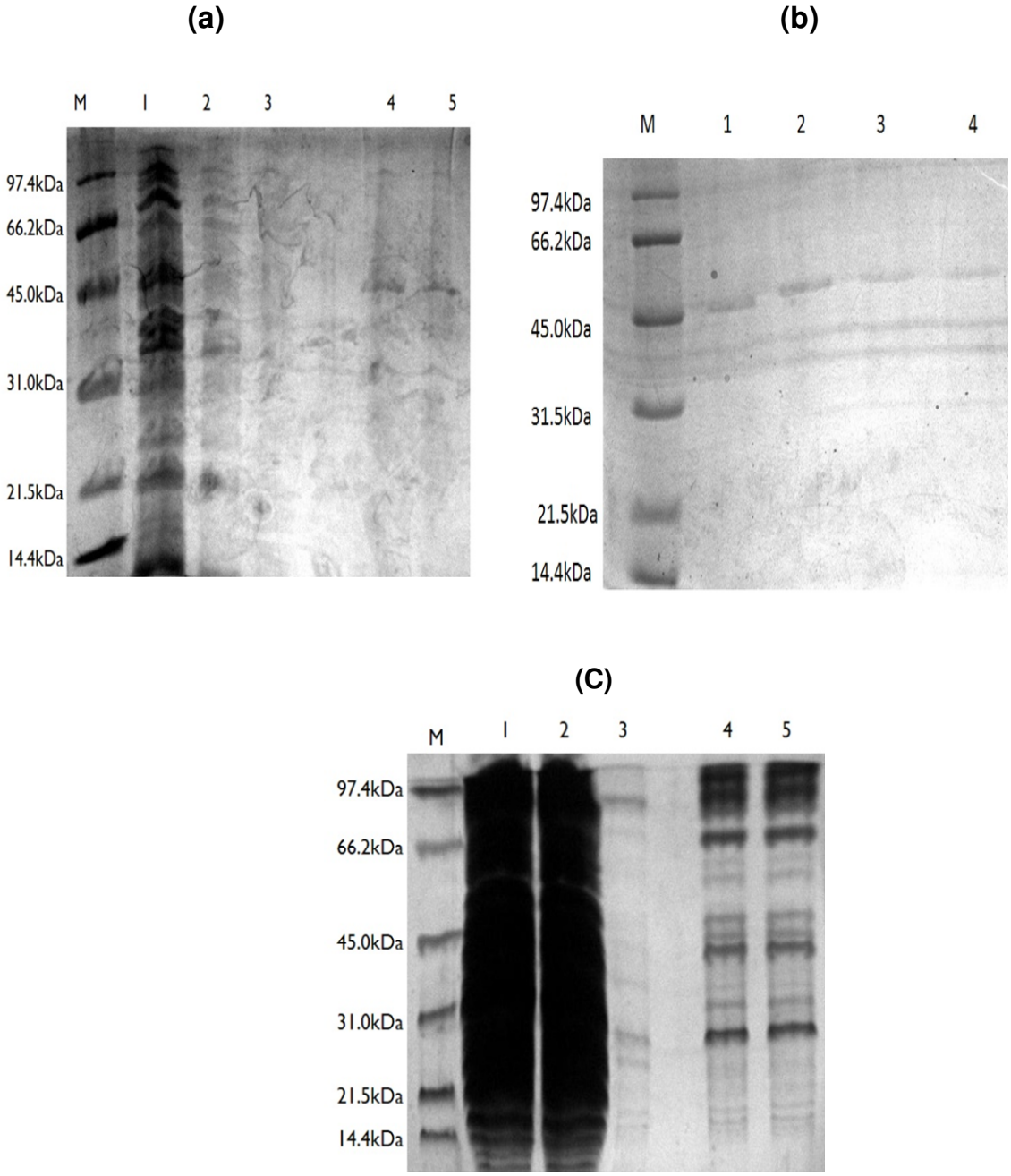

Figure 1. SDS-PAGE analysis of the purification of recombinant bromelain expressed in *E.coli* BL21-AI. (a) SDS-PAGE showing different purification steps of soluble (native) bromelain. Lane M: Protein molecular weight marker; lane 1: Crude cell lysate; lane 2: Flow through of binding step; lane 3: Washing step; lanes 4 and 5: Eluted purified soluble recombinant bromelain. (b) SDS-PAGE showing purified soluble bromelain. Lane M: Protein molecular weight marker; lanes 1, 2, 3 and 4: Purified soluble recombinant bromelain. (C) SDS-PAGE showing different purification steps of insoluble (denatured) bromelain. Lane M: Protein molecular weight marker; lanes 1 and 2: Crude cell lysate; lane 3: Washing step; lanes 4 and 5: Eluted purified insoluble recombinant bromelain.

tertiary structure of the protein was disrupted in the insoluble form. The unfolded proteins (Figure 1c) might have trapped and/or interacted with some proteins during purification process.

The purification results for recombinant bromelain are summarized and shown in Table 1. It can be observed that the purification fold for both native (soluble) and denatured (insoluble) bromelains were 3.7 and 2.7, while their corresponding yield (%) was 64 and 60.5, respectively. This implies that higher purification factor (PF) and

Table 1. Purification of recombinant bromelain of *E. coli* BL21-AI.

Protein fraction	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude (Soluble)	10	2572	41.2	62.4	1.0	100
Purified (soluble)	2.7	1646	7.2	228.6	3.7	64.0
Crude (insoluble)	10	476	26.8	17.8	1.0	100
Purified (insoluble)	2.5	288	5.9	48.8	2.7	60.5

yields were obtained in the soluble form of bromelain. The recovery of soluble protein from inclusion bodies often yield less active enzyme and can significantly raise the cost of bioseparation (Lilie et al., 1998). Consequently, it is vital to express the protein in a soluble and biologically active form. The values of PF and yield of soluble bromelain obtained from this study were found to be slightly higher as compared to 2.11 and 47.5% obtained by Xu *et al*. (2011) for recombinant glycerol dehydratase. In addition to this, Krishna et al. (2011) obtained 2.5 and 46% for recombinant protein (hGMCSF). Both proteins mentioned earlier were purified by using Ni-NTA affinity chromatography technique as well. It could be seen that higher PF and yield values for soluble bromelain were achieved.

Conclusion

It can be concluded that this study had achieved the successful expression of recombinant bromelain. The soluble and insoluble forms of the enzyme were purified to near homogeneity by one-step Ni-NTA His•Bind affinity chromatography. Higher yield and purification factor was attained in the production of soluble form of bromelain. Finally, soluble bromelain of molecular weight of about 45 kDa has been produced.

ACKNOWLEDGEMENTS

The authors are grateful to the MOHE for financial assistance through a Fundamental Research Grant Scheme (FRGS 11-04-0162) and the Department of Biotechnology Engineering, IIUM, for providing all the necessary laboratory facilities to conduct the research work.

REFERENCES

Amid A, Ismail N, Yusof F, Salleh HM (2011). Expression, Purification and Characterization of a Recombinant Stem Bromelain from *Ananas comosus*. Process Biochem. (accepted; in press) PRBI-D-11-00351R1.

Bitange NT, Zhang W, Ying X, Wenbin Z (2008).Therapeutic Application of Pineapple Protease (Bromelain): A review. Pak. J. Nutr. 7(4): 513-520

Bradford MM (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal. Biochem. 72: 248-254.

Dubey VK, Pande M, Singh BK, Jagannadham MV (2007). Papain-like proteases: applications of their inhibitors. Afr. J. Biotechnol. 6(9): 1077-1086.

Gregory S, Kelly ND (1996). Bromelain: A Literature Review and Discussion of its Therapeutic Applications. Alt. Med. Rev. 1(4): 243-257.

Hans PS, Kim KM (2005). Soluble expression of recombinant proteins in the cytoplasm of Escherichia coli. Microb. Cell Fact. 4(1):1-8

Illanes A (2008). Enzyme Production. Enzyme Biocatalysis. Springer Science and Business Media V.B., Brazil, pp. 57-106.

Ismail N, Amid A (2008a). Fast and reliable cloning method for bromelain gene from pineapple (Ananas comosus) stem. International Conference on Science & Technology : Applications in Industry & Education.

Ismail N, Amid A (2008b). Molecular Cloning, Insertion and Transcription Confirmation and Expression Study of Bromelain Gene from *Ananas comosus* Proceedings of the International Graduate on Engineering and Science (IGCES'08)

Krishna MP, Das SB, Shekhar N, Damodaran K, Nair R, Somani S, Veena PR, Jain S, Padmanabhan S (2011). Cloning, Soluble Expression and Purification of High Yield Recombinant hGMCSF in *Escherichia coli*. Int. J. Mol. Sci. *12*: 2064-2076.

Laemmli U (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 237: 680-685

Lilie H, Schwarz E, Rudolph R (1998). Advances in refolding of proteins produced in *E. coli*. Curr. Opin. Biotechnol. 9: 497-501.

Lucia F, Tomas GV (2010). Native and Biotechnologically engineered Plant Proteases with Industrial applications. J. Food Bioprocess Technol. 4: 1066-1088

Lyons TP (1982). Proteinase enzymes relevant to the baking industry. Biochem. Soc. Trans. 10(4): 287-290.

Maria LC, Jose AS, Vanessa V, Lorena A, Maria DM, Maria EH (2008). Expression, Purification and Immobilization of the Intracellular Invertase INVA, from *Zymomonas mobilis* on crystalline Cellulose and Nylon-6. J. Ind. Microbiol Biotechnol 35: 1455-1463

Maurer HR (2001). Bromelain: Biochemistry, Pharmacology and Medical Uses. Cell. Mol. life Sci. 58: 1234-1245.

Paul C (2004). Purification of Therapeutic Proteins. Protein purification Protocols, 2nd edn. Human press, Totowa, New Jersey. pp. 455-475.

Ravindra BB, Rastogi NK, Raghavarao, KS M S (2008). Liquid–liquid extraction of bromelain and polyphenol oxidase using aqueous two-phase system. Chem. Eng. Process, 47: 83-89.

Silverstein RM (1974). The assay of the bromelains using N-CBZ-L-lysine p-nitrophenylester and N-CBZ-L-glycine p-nitrophenyl ester as substrates. Anal Biochem. 62: 478-484.

Theo D (2008). Recombinant Therapeutic proteins: Production Platforms and challenges. Biotechnol. J. 3: 90-97

Xu XL, Zhang GL, Yuan*a* BLYJ, Li C (2011). Recombinant Glycerol Dehydratase from *Klebsiella pneumonia* XJPD: Induction Optimization, Purification and Characterization. Appl. Biochem. Microbiol. 47(2): 144-150.