KMITL Sci. Tech. J. Vol. 11 No. 1 Jan. - Jun. 2011

Optimal Pre-treatment and Resolubilization of Protease B from *Bacillus licheniformis*

Sk. Amir Hossain[1*], Andres Veide[2], Cecilia Janson Kapca[3], Kim Hanson Uhre[3] and Sune Jakobsen[3]

[1*]Biotechnology and Genetic Engineering Discipline, Khulna University, Bangladesh
[2]School of Biotechnology, The Royal Institute of Technology (KTH), Sweden
[3]Department of Recovery Development, Novozymes A/S, Denmark

Abstract

The study was designed to optimize physical parameters (e.g., temperature, maturation time and pH) related to protein solubility in order to reduce the cost of the total process of the enzyme production at industrial scale. We have studied the influence of physical parameters and chemical pre-treatment on the solubility of Protease B in detail. Protease B, a recombinant proteolytic enzyme produced from genetically engineered *Bacillus licheniformis* was used in this study. A longer protein solubility region as a function of pH and dry matter percentage was found at 4°C for Protease B. The maximum dry matter percentage was 20.1% RI (Refractive Index). The optimum protein solubility for Protease B solubility was found between 3.0 to 4.0 mS/cm conductivity within a pH range of 4.0 to 6.0. The solubility trend of the protein was not changed by the level of the enzyme concentration. This study clearly demonstrates that optimization of some physical parameters and chemical pre-treatment improves the solubility of Protease B and thus establishes the conditions of parameters in a cost-effective strategy of Protease B recovery and production at industrial scale.

Keywords: Protein solubility, physical parameters, ultrafiltration concentrate, enzyme concentration, enzyme crystal.

1. Introduction

A general recovery process of industrial enzymes include one or more of the following steps; removal of insolubles, product recovery and isolation e.g., concentration and partial enrichment of products and finally purification or product polishing [1- 3]. In an enzyme production process the minimum desired total protein concentration including product from the culture broth following downstream processing should be around 60 to 70 g/liter [4, 5]. A trouble was reported by Novozymes, Denmark in recovery of Protease B produced by genetically engineered *Bacillus*

*Corresponding author: Mobile: +880-1720478775

E-mail: isti_99@yahoo.com.

licheniformis. The desired enzyme is found insoluble in the form of enzyme crystals in its culture broth. The enzyme crystal goes into sludge during removal of biomass from culture broth by using flocculation, centrifugation or rotary vacuum filtration as a consequence loss of desired enzyme occurs. The problem with crystals in culture broth makes the total recovery process of the enzyme, a low yielding process. Thus to obtain the highest product concentration as well as to keep the production cost low and to keep up yield, protein has to be completely soluble in culture broth and in solution throughout the whole recovery processes.

It is clear that high recovery yield of an enzyme depends largely on the protein solubility [6, 7] which is determined by various interactions between protein-protein, protein-ion, ion-water, and water-protein molecules. Factors associated to crystallization such as temperature, pH, ionic strength, and the presence of stabilizing and denaturing agents in the solution also have great influence on protein solubility [8]. Reducing the rate of protein synthesis by introducing low copy number plasmid such as pProEX^{TM} [9] or changing growth medium with the addition of prosthetic groups or co-factors [8] or mutagenesis [7, 10] may improve the protein solubility. However, these techniques for improving protein solubility increase the cost of the overall process of enzyme production at industrial scale. Therefore, optimization of the physical parameters or chemical pre-treatment related to protein solubility is a better way to reduce the cost of the total process of the enzyme production at industrial scale.

Data on protein solubility first reported in the beginning of the last century [11]. In the first period of the century, solubility data of hemoglobin as a function of the nature of salt versus pH was reported by Green [12, 13]. But data on protein solubility are limited for a wide range of proteins. Therefore, the present study was aimed to optimize different physical parameters and chemical pre-treatment that improve the solubility of Protease B and finally to make a better understanding about the solubility of the enzyme.

2. Materials and Methods

2.1 Enzyme

Protease B ultrafiltrate (UF) concentrate (Novozymes A/S, Denmark) was used throughout the experiments. Protease B is a special kind of protease used in detergent for the purpose of dish washing and is produced by genetically engineered *Bacillus licheniformis*. The enzyme is stabilized by calcium ions. The isoelectric point and the theoretical molecular weight of Protease B is 9.46 and 26797 Dalton respectively.

2.2 Chemicals

NaOH (Gropa A/S, Denmark), CH_3COOH (Bie & Berntsen, Denmark), 34% $CaCl_2$ (Kemira, Denmark), KCl and Na_2SO_4 (J.T Baker, The Netherlands), $MgCl_2.6H_20$ (Merck, Germany) and $CaCl_2.2H_20$ (Sigma–Aldrich, Germany) were used in this study. All chemicals used were of analytical grade. Both tap and deionized water were used in the experiments

2.3 Protease B UF

The initial dry matter percentage, conductivity and enzyme concentration of Protease B UF concentrate were 2.5% RI, 9.24 mS/cm and 162 mg/gm respectively at pH 4.3. Protease B UF concentrate was found to contain a lot of enzyme crystals under microscopic observation. Thus, the pre-treatment of the enzyme solution was needed. After adjustment of pH at 4.0 using 0.1 N CH_3CHOOH solutions, the UF concentrate was diluted two times following addition of deionized water. A lot of enzyme crystals were seen to be present in enzyme solution under microscopic observation. The diluted enzyme solution was further pre-treated by centrifugation at 4500 rev/min

for 20 minutes at 10°C and with filtration by 0.2 microfilter (Sartorius). The filtrated centrifuged supernatant was used for further experiments as starting material.

2.4 Concentrating Protease B UF

After minimal pre-treatment of the Protease B UF, it was further concentrated using a cross flow filtration system (Sartorius Corporation, USA) at ambient temperature up to 28.4% RI with no signs of precipitation. In order to measure the dry matter percentage, and to observe the physical parameters *e.g.* haze, precipitate and clarity of the UF concentrate, a sample of 10 ml concentrate was taken out with approximately 2% RI increase starting from 8.4% to 28.4% RI. The dry matter percentage and enzyme conductivity were measured by a Refractometer (Bellingham and Stanley Ltd., UK) and by Conductivity meter (Radiometer A/S, Denmark) respectively. Samples were then left to stand both at 4˚C and ambient temperature (20-22˚C) for 12 hours for further investigation.

2.5 UF Diawash

After pre-treatment, the filtrated supernatant of Protease B UF concentrate with 20.1% RI was then diawashed down from 5.8 to 3.0 mS/cm. A sample of 100 ml was taken at three conductivity steps (5.8, 5.0, 4.0 and 3.0 mS/cm) during the diawash. A volume of 10 ml enzyme filtrate from each sample was portioned out in small beakers. The pH of the enzyme filtrate was then adjusted from 4.0 to 6.0 with an inclement of 0.4 units using 0.1 N NaOH solutions. A sample of 5 ml of each 10 ml enzyme filtrated supernatant at the same pH was then left at both 4˚C and ambient temperature (20-22˚C) and examined after 12 hours.

2.6 Microtiter plate assay

Microtiter plates with 96 wells were used in these experiments. The UF concentrate at optimal condition were added using microtiter pipette into the first 12 wells at the same desired enzyme concentration but at different pH values. An appropriate volume of water according to desired dilution rate with the same pH values was then added to A-H well in a total volume of 250 µl solution. The microtiter plate was then left at ambient temperature (20-22°C) and examined after 12 hours.

3. Results and Discussion

3.1 Measurement of maximum dry matter percentage (%RI)

The centrifuged supernatant was used as the starting material in this experiment. The centrifuged supernatant looked clear with a dry matter percentage of 8.0 % RI. The supernatant was further concentrated up to 22.5% RI on a concentration device without showing precipitation. The maximum dry matter percentage was 22.5% RI. All of the 12 samples were left at 4˚C and examined after 12 hours. All the samples showed no haze and precipitate. Therefore, the maturation time showed significant effect on protein solubility. The experiment was repeated with further pre-treated Protease B UF concentrate following by filtration. The UF filtrate was then concentrated up to 30.9% RI on a concentration device at ambient temperature without showing precipitation. All the samples were then left both at ambient temperature (20-22˚C) and 4˚C for 12 hours to observe the effect of temperature on protein solubility. All data obtained from this experiment are shown in the Tables 1 and 2.

Table 1. Effect of dry matter percentage (% RI) on the optimal solubilization of Protease B UF at 4°C and pH 4.0 after 12 hours.

Samples	Concentrate RI (%)	Physical Properties
01	8.0	NH,NP,C+
02	11.5	NH,NP,C+
03	14.3	NH,NP,C+
04	16.5	NH,NP,C+
05	19.7	NH,NP,C+
06	22.6	NH,NP,C+
07	25.1	LH,NP,C
08	28.3	LH,NP,C
09	30.9	H,NP,C

Note: NH: No Haze, LH: Little Haze, H: Haze, NP: No Precipitate, **P** = Precipitate, C: Clear to some extent, C+: Clear to great extent.

Table 2. Effect of dry matter percentage (% RI) on the optimal solubilization of Protease B filtered supernatant at ambient temperature (20-22˚C) and pH 4.0 after 12 hours.

Samples	Concentrate RI (%)	Physical Properties
01	8.0	NH,NP,C+
02	11.5	NH,NP,C+
03	14.3	NH,NP,C+
04	16.5	NH,NP,C+
05	19.7	NH,NP,C+
06	22.6	LH,NP,C
07	25.1	LH,NP,C
08	28.3	H,NP,C
09	30.9	**P**

Note: NH: No Haze, LH: Little Haze, H: Haze, NP: No Precipitate, **P** = Precipitate, C: Clear to some extent, C+: Clear to great extent.

The solution of enzyme could hold maximum dry matter percentage around 20% both at 4˚C and ambient temperature. The maturation time showed significant effect on protein solubility. The sterile filtration was found to increase the protein solubility with respect to dry matter percentage.

3.2 Measurement of optimum pH and conductivity on enzyme solubility

Protease B filtrate with 20.1% RI at pH 4.0 was used as the study material in this experiment. The conductivity of Protease B filtrated supernatant was 5.8 mS/cm. The conductivity of Protease B filtrated supernatant was lower than that of the Protease B UF concentrate because of filtration with 0.2 μm filter. This indicates that not only microorganisms but also some of the salts were removed following sterile filtration. The filtrated supernatant with 20.1% RI was then diawashed down from 5.8 to 3.0 mS/cm. All data obtained from these experiments are given in the Tables 3 and 4.

Table 3. Effect of modification in pH and conductivity on optimal solubilizaiton of Protease B filtered concentrate at 4˚C with 20.1% RI after 12 hours.

pH	**Conductivity (mS/cm)**			
	3.0	4.0	5.0	5.8
4.0	NH,NP,C+	NH,NP,C+	NH,NP,C+	NH,NP,C+
4.4	NH,NP,C+	NH,NP,C+	NH,NP,C+	NH,NP,C+
4.8	NH,NP,C+	NH,NP,C+	NH,NP,C+	NH,NP,C+
5.2	NH,NP,C+	NH,NP,C+	NH,NP,C+	NH,NP,C+
5.6	NH,NP,C+	NH,NP,C+	NH,NP,C+	LH,NP,C
6.0	NH,NP,C+	NH,NP,C+	LH,NP,C	LH,NP,C

Note: NH: No Haze, LH: Little Haze, H: Haze, NP: No Precipitate, **P**: Precipitate, C: Clear to some extent, C+: Clear to great extent.
The UF concentrate was diawashed down from 5.8 to 4.0 mS/cm by adding a required amount of deionized water. The conductivity of the deionized water was 6.0 μS/cm.

Table 4. Effect of modification in pH and conductivity on optimal solubilizaiton of Protease B filtered concentrate at ambient temperature (20-22˚C) with 20.1% RI after 12 hours.

pH	**Conductivity (mS/cm)**			
	3.0	4.0	5.0	5.8
4.0	NH,NP,C+	NH,NP,C+	NH,NP,C+	NH,NP,C+
4.4	NH,NP,C+	NH,NP,C+	NH,NP,C+	NH,NP,C+
4.8	NH,NP,C+	NH,NP,C+	NH,NP,C+	H,NP,C
5.2	LH,NP,C	LH, NP,C	H,NP,C	**P**
5.6	**P**	**P**	H,NP,C	**P**
6.0	**P**	**P**	**P**	**P**

Note: NH: No Haze, LH: Little Haze, H: Haze, NP: No Precipitate, **P**: Precipitate, C: Clear to some extent, C+: Clear to great extent. The UF concentrate was diawashed down from 5.8 mS/cm to 3.0 mS/cm by adding a required amount of deionized water. The conductivity of the deionized water was 6.0 μS/cm.

The result of this experiment shows both salting-in and salting-out region. Therefore, the solubility of Protease B UF concentrate is largely dependent on both ionic strength and pH. A

sample of enzyme concentrate at pH 5.2 was taken for further experiments as the optimum pH with conductivity 5.8 mS/cm and 20.1% RI at 4ºC. The result shows that temperature, pH, conductivity and maturation time have significant effect on protein solubility and the lower temperature e.g. 4˚ C is preferable for enzyme solubility.

3.3 Measurement of optimal conditions of enzyme solubilisation

Protease B filtrated supernatant having 20.1% RI, 5.8 mS/cm conductivity at pH 5.2 was used as the starting material in the following experiment. The initial enzyme concentration in the filtrated concentrate was 114 mg/g. Both of the microtitier plates were left at ambient temperature (20-22˚C) and examined after 12 hours. The enzyme solution in the entire well of both of the microtitier plates showed no haze and precipitate. Therefore, one more experiment was conducted with 80.4 mg/g enzyme concentration. Enzyme concentrate with all dilution rates except 100% dilution was found to show clear solutions with no precipitate and haze over a large range of pH 4.0 to 6.0. Therefore, the experiment was repeated with Protease B filtrated concentrate with 57.45 mg/g enzyme concentration. All data obtained from this experiment are shown in Figure 1. Enzyme crystals were soluble at pH 8.0 with the highest enzyme concentration (80.42 mg/g). The solubility of Protease B UF concentrate was found within 80.42 to 40.22 mg/g enzyme concentration over a range of pH 4.0 to 8.0. The protein solubility was noted at pH 8.0 which is near the isoelectric point of Protease B and the solubility was found to increase with decreased pH values.

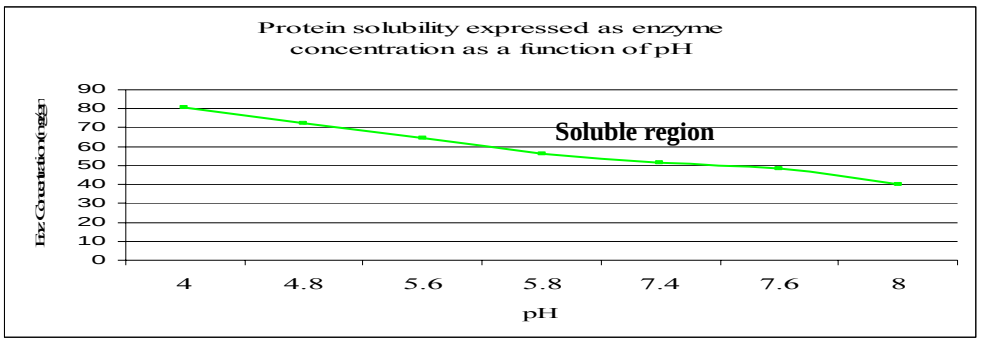

Figure 1 Protease B solubility is expressed as enzyme concentration as a function of pH. The solubility region was shown with different enzyme concentration within a pH range of 4.0 to 8.0.

4. Conclusions

The present research demonstrated a complete solubility pattern of Protease B. A minimal pre-treatment including centrifugation and sterile filtration was needed for Protease B in order to prepare the starting material for the determination of Protease B solubility behavior. Sterile filtration using 0.2 μm filter could be effective in reducing microbial growth by removing microorganisms in the starting material. Though, no microbial test was conducted in order to find out the level of microorganisms in the enzyme solutions before and after filtration. But the solubility range of Protease B with respect to dry matter percentage was found to increase following sterile filtration. An additional pre-treatment including centrifugation and sterile filtration is needed to prepare Protease B UF concentrate as the starting material used in this research work. Thus, the idea about this pre-treatment for Protease B could be helpful in early downstream processes at large scale Protease B production by Novozymes, Denmark.

Factors that usually controls protein solubility e.g., temperature, maturation time and pH were found to have significant effect on Protease B solubility. In case of temperature, a longer protein solubility region as a function of pH and dry matter percentage was found at 4°C for Protease B than that at ambient temperature. The maximum dry matter percentage of the enzyme was determined as 20.1% RI at pH 4.0. The optimum Protease B solubility was noted between 3.0 to 4.0 mS/cm conductivity within a pH range, 4.0 to 6.0. The idea about the optimum dry matter percentage, conductivity and pH ranges with optimum protein solubility for the enzyme could help to keep all the crystals soluble in enzyme solution in all the unit operations of downstream processes for Protease B used by Novozymes, Denmark in order to avoid enzyme loss during recovery of industrial enzymes.

The solubility region of Protease B with respect to enzyme concentration was found within the range of 80.4 to 32.7 mg/g over a wide range of pH starting from 4.2 to 8.0. The solubility trend of the protein was found to be unchanged by the level of the enzyme concentration. The idea about the Protease B solubility region as a function of pH with respect to enzyme concentration could help maintain the enzyme concentration in the entire downstream processes for Protease B recovery by Novozymes, Denmark. In order to keep all the enzyme crystals soluble in enzyme solution as a consequence the possibility of getting highest enzyme concentration in the final product will be increased. This will help reduce the recovery cost of Novozymes, Denmark which ultimately reduce the total production cost of Protease B.

5. Acknowledgements

I thank the Department of Recovery Development, Novozymes A/S, Denmark for supporting my fundamental research on the basis of protein solubility of Protease B. I would like to express my deep sense of gratitude and indebtedness to Peter Klein for his kind cooperation during my research work.

References

[1] Belter, P. A., Cussler, E. L. and Hu, W.-S. **1988**. *Bioseparations: Downstream Processing for Biotechnology,* John Wiley & Sons, New York.

[2] Dwyer, J. L. **1984**. *Bio/Technology*, 2, 957.

[3] Bonnerjea, J., Oh, S., Hoare, M., and Dunnill, P. **1986**. *Biotechnology, 4*, 954.

[4] Asenjo, J. A. **1988**. The rational design of large scale protein separation sequences. *Paper presented at the 196th ACS National Meeting,* MBTD division, Los Angeles, 25-30.

[5] Pharmacia, **1983**. Scale up to process chromatography. Guide to Design, *Pharmacia,* Uppsala, Sweden.

[6] Faber, C., Hobley, T., Mollerup, J., Thomas, O., Kaasgaard, S. **2008.** Factors affecting the solubility of *Bacillus halmapalus* α-amylase. *Chem. Eng. Process*. 47,1007-1017.

[7] Saul, R. T., Scholtz, J. M., Pace, C. N., **2008**. Measuring and increasing protein solubility. *J. Pharm.* Sci. 97,4155-4166.

[8] Retailleau, P., Ries-Kautt, M., Ducruix, A. **1997**. No salting-in of lysozyme chloride observed at low ionic strength over a large range of pH. *Biophys. J.* 71, 2156-2163.

[9] Robert, A., Donahue, Jr., Bebee, R. L. **1999**. BL21-SI competent cells for protein expression in *E. coli. Focus.* 21, 49-51.

[10] Liu, J. W., Boucher, Y., Stokes, H. W., Ollis, D. L. **2006**. Improving protein solubility: The use of the *Escherichia coli* dihydrofolate reductase gene as a fusion reporter. *Prot. Expr. Purr*. 47, 258-263.

[11] Green. A. A. **1932**.Studies in the physical chemistry of the proteins. *Phys. Chem. Protein.* 10, 47-66.

[12] Green, A. **1931**. Studies in the physical chemistry of the proteins. VIII. The solubility of hemoglobin in concentrated salt solutions: a study of the salting-out of proteins. *J. Biol. Chem.* 93, 495-516.

[13] Green, A. **1931**. Studies in the physical chemistry of the proteins. IX. The effect of electrolytes on the solubility of hemoglobin in solutions of varying hydrogen ion activity with a note on the comparable behavior of casein. *J. Biol. Chem.* 93, 517-542.