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OPTIMIZATION OF PHYSICAL PARAMETERS AND CHEMICAL PRE-TREATMENT IMPROVES THE SOLUBILITY OF PROTEASE A, A RECOMBINANT PROTEOLYTIC ENZYME

Sk. A. Hossain[1]*, A. Veide[2], C.J. Kepka[3], K. H. Uhre[3], and S. Jakobsen[3]

[1]*Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna 9208, Bangladesh*
[2]*School of Biotechnology, The Royal Institute of Technology (KTH), Sweden*
[3]*Department of Recovery Development, Novozymes A/S, Denmark*

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Abstract: Cost of total process of enzyme production at industrial scale depends mainly on its recovery and the recovery largely depends on the solubility of enzymes in solution. Protease A, a proteolytic enzyme produced by genetically engineered *Bacillus licheniformis* is included in the detergents used for dish washing. In the present study, we have studied the influence of physical parameters and chemical pre-treatment on the solubility of Protease A in detail. 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 A than that of ambient temperature. The maximum dry matter percentage was found 20.4% RI at pH 4.0. The optimum protein solubility for Protease A was found with in a conductivity range, 4.0 to 5.0 mS/cm within a pH values 4.0 to 6.0 with the highest clarity. The solubility region of Protease A with respect to enzyme concentration was found within the range of 81.0 to 35.4 mg/gm over a large range of pH starting from 4.0 to 8.0. The solubility trend of the protein was found not to be changed by the level of the enzyme concentration. Findings of this study followed the theory related to protein solubility. Pre-treatment with Proxel, an antimicrobial agent increased the solubility of Protease A as a function of pH. This study clearly demonstrates that optimization of some physical parameters and chemical pre-treatment improves the solubility of Protease A and thus establishes the conditions of parameters in a cost-effective strategy of Protease A recovery and production at industrial scale.

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

Introduction

A general recovery process of industrial enzymes include one or more of the following steps; removal of insoluble, product recovery and isolation e.g., concentration and partial enrichment of products and finally purification or product polishing (Belter *et al*., 1988; Dwyer, 1984; Bonnerjea *et al.,* 1986). 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/litre (Asenjo, 1988; Pharmacia, 1983). But, the target total protein concentration in the final product should be as high as possible to be achieved. A trouble was reported by Novozymes, Denmark in recovery Protease A produced by genetically engineered *Bacillus 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

*Corresponding author: <isti_99@yahoo.com>

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broth by using flocculation, centrifugation or rotary vacuum filtration. Finally, loss of desired enzyme occurs as it is not completely soluble in the solutions while concentration and purifying the products. The problem with crystals in culture broth makes the total recovery process of the enzyme, a low yielding process and creates problem on ultrafiltraiton as well. Thus to obtain the highest product concentration to reach the highest product concentration (more than 60 to 70 g/litre) 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 solutions throughout the whole recovery processes.

It is clear that high recovery yield of an enzyme depends largely on the protein solubility and therefore it is important to know the parameters controlling the solubility of protein (Faber, 2007). Protein solubility is determined by various interactions between protein-protein, protein-ion, ion-water, and water-protein molecules. Protein properties affecting solubility are proteins net charge, the ratio of charged, polar and neutral amino acids, hydrophobicity or hydrophilicity and overall stability versus temperature, pH and solvent compounds. 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 (Retailleau *et al.,* 1997).

Reducing the rate of protein synthesis by introducing low copy number plasmid such as pProEX^{TM} (Robert *et al.,*1999) or changing growth medium with the addition of prosthetic groups or co-factors (Robert *et al.,* 1999) or mutagenesis (Liu *et al*., 2006) may improve the protein solubility. However, these techniques to get improved 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 (Green, 1932). 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, 1931a, b. But data on protein solubility are limited for a wide range of proteins. Protease A 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 A is 9.69 and 26698 Dalton respectively. The present study was aimed to optimize different physical parameters and chemical pre-treatment that improve the solubility of Protease A and finally to make a better understanding about the solubility of the enzyme.

Materials and Methods

Enzyme: Protease A ultrafiltrate (UF) concentrate (Novozymes A/S, Denmark) were used as the study enzyme throughout the experiments.

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), $CaCl_2.2H_20$ (Sigma–Aldrich, Germany), LiCl and NaCl (Acros, USA), Proxel LV (Avecia, UK). Trizma (Sigma-Aldrich, Germany), and casein (EMD Biosciences Inc., Germany) DL-Apfelsauce (Merck Schuchardt OHG, Germany) and litex agarose (Lonza Copenhagen A/S, Denmark) were used in this study. Proxel LV is an aqueous solution of 1,2-enzisothaiazoline-3-1 and has been used to control microorganisms in industrial fluids for a long time. All chemicals used in this study were of analytical grade. Both tap and deionized water were used in the experiments

Protease A UF: The initial dry matter percentage, conductivity and enzyme concentration of Protease A UF concentrate were 8.4% RI, 9.4 mS/cm and 41-46 mg/gm respectively. The UF concentrate looked clear with no haze and precipitate. The UF concentrate was then adjusted to

pH 4.0. As the Protease A UF concentrate was found clear with no sign of precipitation, no more pre-treatment of Protease A UF concentrate was required. The refractive index (RI) of a substance is a measure of the speed of light in that substance. It is a fundamental physical property of a substance and is often used to identify a particular substance, confirm its purity, or measure the dry matter present in that substance.

Concentrating Protease A UF: After minimal pre-treatment of the Protease A UF, concentrate was further concentrated using a Sartorius 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, RFM110 (Bellingham and Stanley Ltd., UK) and by Conductivity meter (Radiometer A/S, Denmark) respectively. While increasing the concentration up to 28.4% RI, no precipitate was observed in the UF concentrate of all samples taken. Samples were then left to stand at ambient temperature (20-22˚C) for 12 hours and studied again.

UF Diawash: Protein A UF concentrate with 8.4% RI and 9.4 mS/cm conductivity, 40 - 46 mg/gm and pH 4.6 was used as study material in this experiment. The UF concentrate was found to show no haze and precipitate with the highest clarity. After pre-treatment, the Protease A UF concentrate was concentrated further up to the maximum dry matter percentage 20.4 % RI on a UF concentration device that was determined in the first experiment. The UF concentrate was then diawashed down by using a cross flow filtration system (Sartorius Corporation, USA) to approximately 4 mS/cm following by addition of required amount of deionised water. A sample of 100 ml was taken out at 7.3, 7.0, 6.0, 5.0 and 4.0 mS/cm and portioned out in small beakers. A volume of 10 ml UF concentrate was portioned out from each 100 ml sample into small beakers. Then, the pH was adjusted from 4.0 to 6.0 with an incline of 0.4 pH units in each beaker and was kept at 4˚C and studied again after 12 hours.

Microtiter plate assay: Important parameters (e.g. dry matter percentage, conductivity, enzyme concentration and pH etc.) associated with the solubility of the enzyme can be studied using mirotiter 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 studied again after 12 hours. Different physical characteristics (e.g. haze, precipitate and clarity) of enzyme solution in each well were noted down.

Results

Measurement of maximum dry matter percentage (%RI): The following experiment was conducted in order to find out maximum dry matter (RI %) percentage that the UF concentrate could contain with no signs of precipitation. Protease A UF concentrate was used as the study material in this experiment. Following after pre-treatment and UF concentration as described in the materials and methods section, all the samples were then left at ambient temperature (20-22˚C) at pH 4.0. All the data obtained from the experiment are shown in the Table 1.

Table 1. Effect of dry matter percentage (% RI) on the optimal solubilizaiton of Protease A UF at ambient temperature (20-22°C) and pH 4.0

Samples	*Concentrate RI (%)*	*Physical Properties*
1	8.4	NH,NP,C+
2	11.5	NH,NP,C+
3	14.6	NH,NP,C+
4	16.7	NH,NP,C+
5	19.1	NH,NP,C+
6	21.1	H,NP,C
7	22.2	P
8	23.2	P
9	23.9	P
10	25.2	P
11	24.1	P
12	25.5	P
13	27.0	P
14	28.4	P

NB: NH: No Haze, H: Haze, NP: No Precipitate, P = Precipitate after 12 hours, C: Clear to some extent, C+: Clear to great extent. Concentration was done using Sartorius cross flow filtration system at ambient temperature (20-22˚C).

Effect of pH and Proxel pre-treatment on enzyme solubility: The following experiment was conducted to determine the optimum pH to keep the protein in solution in order to get optimum yield. An antimicrobial agent (Proxel) was used in the experiment to observe the effect of Proxel on protein solubility. Pre-treated Protease A UF concentrate (as described in the materials and methods section) was used as the starting material in the experiments. Three microtitier plate assays were conducted. First two experiments were performed in absence and in presence of Proxel (2 drops in the starting material). UF concentrate with 8.4% RI was used as the starting material in these experiments. While the third experiment was performed with UF concentrate (21.1% RI) in presence of Proxel (2 drops in the starting material). Table 2 shows the results that were obtained from the experiment conducted with the UF concentrate containing 8.4% RI in absence of Proxel.

Table 2. Effect of pH modification with dilution of dry matter (8.4% RI) on the optimal solubilization of Protease A UF in absence of Proxel at ambient temperature (20-22°C) after 12 hours

Dilution rate (% v/v)	*Dry matter (%RI)*	*pH*								
		4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0
100	8.40	NH,NP,C+	NH,NP,C+	P	P	P	P	P	P	P
90	7.56	NH,NP,C+	NH,NP,C+	P	P	P	P	P	P	P
80	6.72	NH,NP,C+	NH,NP,C+	H,NP,C	P	P	P	P	P	P
70	5.88	NH,NP,C+	NH,NP,C+	H,NP,C	H,NP,C	P	P	P	P	P
60	5.04	NH,NP,C+	NH,NP,C+	H,NP,C	H,NP,C	P	P	P	P	P
40	4.20	NH,NP,C+	NH,NP,C+	H,NP,C	H,NP,C	P	P	P	P	P
30	3.36	NH,NP,C+	NH,NP,C+	H,NP,C	H,NP,C	P	P	P	P	P
10	0.84	NH,NP,C+	NH,NP,C+	NH,NP,C+	H,NP,C	P	P	P	P	P

NB: NH: No Haze, NP: No precipitate, H: Haze, **P**: Precipitate, C: Clear to some extent, C+: Clear to great extent

The next experiment was conducted with Protease A UF concentrate containing the same dry matter (8.4% RI) percentage in presence of Proxel. The experiment was performed at ambient temperature (20-22˚C). In this case, a broader enzyme optimum pH range with respect to enzyme solubility (Table 3) from 4.0 to 5.5 was found with a dilution of the concentrate up to 90%.

Table 3**.** Effect of pH modification with dilution of dry matter (8.4% RI) on the optimal solubilization of Protease A UF in presence of Proxel at ambient temperature (20-22°C) after 12 hours

Dilution rate (% v/v)	*Dry Matter (%RI)*	*pH* 4.0	4.5	5.5	6.0	6.5	7.0	7.5
100	8.40	NH,NP,C+	P	P	P	P	P	P
90	7.56	NH,NP,C+	NH,NP,C+	NH,NP,C+	H,NP,C	P	P	P
80	6.72	NH,NP,C+	NH,NP,C+	NH,NP,C+	H,NP,C	P	P	P
70	5.88	NH,NP,C+	NH,NP,C+	NH,NP,C+	H,NP,C	H,NP,C	P	P
60	5.04	NH,NP,C+	NH,NP,C+	NH,NP,C+	H,NP,C	H,NP,C	P	P
40	4.20	NH,NP,C+	NH,NP,C+	NH,NP,C+	H,NP,C	H,NP,C	H,NP,C	P
30	3.36	NH,NP,C+	NH,NP,C+	NH,NP,C+	NH,NP,C+	H,NP,C	H,NP,C	H,NP,C
10	0.84	NH,NP,C+	NH,NP,C+	NH,NP,C+	NH,NP,C+	NH,NP,C+	H,NP,C	H,NP,C

NB: NH: No Haze, NP: No precipitate, **P**: Precipitate, H: Haze, C: Clear to some extent, C+: Clear to great extent.

An experiment more was conducted at ambient temperature (20-22˚C) with higher dry matter percentage (21.1% RI) in presence of Proxel to observe the effect of dry matter percentage on protein solubilisation. Then the microtiter plate was left at ambient temperature (20-22˚C) and studied again after 12 hours. The UF concentrate with 21.1% RI was taken as the starting material in this experiment to observe the effect of Proxel as well as dry matter percentage on protein solubility. All of the data obtained from this experiment are shown in the Table 4.

Table 4**.** Effect of pH modification with dilution of dry matter (21.1% RI) on the optimal solubilization of Protease A UF in presence of Proxel at ambient temperature (20-22°C) after 12 hours.

Dilution rate (% v/v)	*Dry matter (% RI)*	*pH* 5.0	5.5	6.0	7.0	8.0
1.0	21.10	P	P	P	P	P
0.9	18.99	P	P	P	P	P
0.8	16.88	P	P	P	P	P
0.7	14.77	P	P	P	P	P
0.6	12.66	P	P	P	P	P
0.4	10.55	P	P	P	P	P
0.3	6.33	H,NP,C	H,NP,C	P	P	P
0.1	2.11	H,NP,C	H,NP,C	P	P	P

NB: NH: H: Haze, NP: No precipitate, **P**: Precipitate, C: Clear to some extent.

Measurement of optimum pH and conductivity on enzyme solubility: Protease A UF concentrate with 8.4% RI and 9.4 mS/cm conductivity, 41 - 46 mg/g and pH 4.6 was used as study material in this experiment. First of all, the concentrate was adjusted to pH 4.0. Then the UF concentrate was concentrated up to the maximum dry matter percentage 20.4 % RI on a UF concentration device that was determined in the first experiment. The UF concentrate was then diawashed down to approximately 4 mS/cm as discussed in the materials and methods section. A sample of 100 ml was taken out at five different conductivity steps (7.3, 7.0, 6.0, 5.0 and 4.0 mS/cm) and portioned out in small beakers was kept at 4˚C and studied again after 12 hours. From this experiment, the optimum protein solubility was found with in a conductivity range, 4.0 to 5.0 mS/cm within a pH values 4.0 to 6.0 without any precipitate in the enzyme solutions along with the highest clarity. Then, the solubility was found to decrease with increased conductivity. All the data obtained from this experiment are shown in the Table 5.

Table 5**.** Effect of modification in pH and conductivity on optimal solubilizaiton of Protease A UF at 4°C with 20.4% RI.

pH	Conductivity (mS/cm)				
	4.0	5.0	6.0	7.0	7.3
4.0	NH,NP,C+	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+	NH,NP,C
4.8	NH,NP,C+	NH,NP,C+	NH,NP,C+	LH,NP,C	NH,NP,C
5.2	NH,NP,C+	NH,NP,C+	LH, NP,C	LH,NP,C	LH,NP,C
5.6	NH,NP,C+	NH,NP,C+	NH,NP,C	LH,NP,C	H,NP,C
6.0	NH,NP,C+	NH,NP,C+	LH, NP,C	H,NP,C	LH,NP,C

NB: NH: No Haze, LH: Little Haze, H: Haze, NP: No Precipitate, C: Clear to some extent, C+: Clear to great extent. The UF concentrate was diawashed down from 9.4 mS/cm to 4.0 mS/cm by adding a required amount of tap water.

Measurement of optimal conditions of enzyme solubilisation: The following experiment was performed to study optimal conditions of enzyme solubilisation. Protease A UF concentrate with 20.4% RI and 7.0 mS/cm conductivity at pH 4.4 and 4˚ C was found to show optimal solubility in solution of enzyme (Table 5). Thus, the UF concentrate with 20.4% RI, 7.0 mS/cm conductivity at pH 4.4 was used as study material in the following experiment. The enzyme concentration in the taken UF concentrate was 112 mg/g. First of all, preconditioned water with 7 mS/cm conductivity was prepared by adding required amount of 34% (w/w) $CaCl_2$ to deionised water. The UF concentrate was diluted with preconditioned water to adjust the enzyme concentration; 30.4 mg/g in the UF concentrate in order to keep the conductivity constant at different pH values. The microtitier plates were then left at ambient temperature (20-22˚C) for 12 hours. All the data obtained from this experiment is shown in the Table 6.

Table 6: Effect of modification in pH and conductivity on optimal solubilizaiton of Protease A UF at ambient temperature (20-22˚C) with 20.4% RI

pH	Conductivity (mS/cm)				
	4.0	5.0	6.0	7.0	7.3
4.0	NH,NP,C+	LH,NP,C	LH,NP,C	LH,NP,C	H, NP, C
4.4	NH,NP,C+	LH,NP,C	LH,NP,C	LH,NP,C	H, NP, C
4.8	NH,NP,C+	LH,NP,C	LH,NP,C	H, NP, C	H, NP, C
5.2	LH,NP,C	LH,NP,C	LH,NP,C	H, NP, C	H, NP, C
5.6	LH,NP,C	LH,NP,C	LH,NP,C	**P**	**P**
6.0	LH,NP,C	LH,NP,C	LH,NP,C	**P**	**P**

NB: 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 9.4 mS/cm to 4.0 mS/cm by adding a required amount of tap water.

Discussion

It is clear that the percentage of dry matter, maturation time and pH have significant effect on protein solubility. The antimicrobial agent, Proxel was found effective in increasing enzyme solubility as a function of pH and dry matter percentage in enzyme solution. The dry matter percentage was low (8.4% RI) (Table 3). However treatment with Proxel did not influence protein solubility in presence of higher dry matter percentage (21% RI) (Table 4) when the solubility was considered as a function of pH and dry matter percentage. Thus, the effectiveness of Proxel was found to depend on dry matter percentage present in enzyme solution. In presence of lower dry matter percentage, the enzyme solubility could be increased by reducing microbial growth using Proxel. Though, no microbial test was conducted in order to determine the level of microbial growth to make sure that whether the microbial growth is responsible for reducing the solubility level or not. Thus, it could be a good way to perform a microbial test in order to make sure whether the experiment was affected by microbial growth or not with respect to enzyme solubility.

The enzyme solution was taken to the conductivity 7 mS/cm (Table 5). This is because the conductivity 7 mS/cm is near to initial conductivity 9.4 mS/cm of the Protease A UF concentrate (starting material of this experiment) that requires the minimum diawash of the starting UF concentrate to reach. pH 4.0 is considered as the lowest pH where the enzyme can regain the complete folding structure in solutions. That is why a sample of enzyme concentrate at pH 4.4 was taken for further experiments as the optimum pH with conductivity 7.0 mS/cm at 4º C with 20.4% RI.

The same experiment was conducted with the same study material and samples were kept at ambient temperature (20-22˚C) to observe the effect of temperature on enzyme solubility (Table 6). The same pH and conductivity range were used in this experiment in order to find out the effect of temperature. Each enzyme solution was found to either precipitate or haze within the conductivity of 5.0 to 7.3 mS/cm at a broader pH ranges from 4.0 to 6.0. Enzyme solution with the lowest conductivity (4.0 mS/cm) was found to show highest clarity having no precipitate within a short pH range of 4.0 to 4.8.

A large Protease A solubility range as a function of pH and conductivity at 4˚ C was found while the solubility range at ambient temperature became small. Most of the enzyme samples with a conductivity range 4.0 to 7.3 mS/cm over a large range of pH starting from 4.0 to 6.0 was found clear with no sign of precipitation following just after diawash and adjustment of pH. But when all the samples were kept standing for 12 hours both at ambient temperature and 4˚C, the enzyme solubility level was found to be affected significantly. The effect was found higher at ambient temperature than that of 4˚C. Microbial growth could be responsible for this kind of a big difference in protein solubility between ambient temperature and 4˚C. Although, no microbial test was performed to make sure whether the growth of microbes was responsible for this kind of effect or not. In conclusion, temperature, pH, conductivity and maturation time have significant effect on protein solubility and the lower temperature e.g. 4˚ C is preferable for enzyme solubilisation of enzymes.

Solutions of enzyme in the entire microtiter plate did not show any haze and precipitate with the highest clarity. As the solutions of enzyme in the entire microtitier plate did not show any haze and precipitate, the experiment was repeated with higher enzyme concentration of 50.6 mg/g. Again, enzyme solution with no haze and precipitate was found in the entire well (A-H) in

both of the microtiter plates except within a dilution rate, 100 and 90% at pH 8.0. Solutions of enzyme with dilution rate, 100 and 90% corresponds to 50.6 mg/g and 45.7 mg/g enzyme concentration respectively were found to show haze with less clarity. Therefore, the experiment with 65.8 mg/g g enzyme concentration was reconducted. Enzyme solution was found to be cleand having no precipitate within a broader pH range of 4.0 to 7.0 and from 7.4 to 7.8. Precipitate was found at pH 7.2 and 8.0 within a dilution rate of 80 to 10%. Enzyme solutions became hazy at pH 7.2 and 8.0 within a dilution rate of 40 to 70%. During the adjustment of pH at 7.2 of the enzyme concentrate, a drop of 37% NaOH was added instead of diluted NaOH. That's why the enzyme concentrate in well was more concentrated at pH 7.2 than in other wells which make the enzyme concentrate precipitate within a dilution rate of 80 to 10% and haze within a dilution rate of 70 to 40%. Thus, the result at pH 7.2 was avoided in this experiment.

The Fig. 1 shows the protein solubility region of Protease A with respect to enzyme concentration as a function of pH. Enzyme crystals were found soluble at pH 4.2 with the highest enzyme concentration of 81.0 mg/g. The enzyme solubility as a function of pH was found to increase with decreased enzyme concentration. The enzyme solubility of Protease A UF concentrate was found within a decreasing range of enzyme concentration from 81.0 to 35.44 mg/g over a large range of pH 4.2 to 8.0. The solubility of Protease A with respect to enzyme concentration as a function of pH decreased with increased pH starting from 4.2 to 8.0. Thus the lowest solubility with respect to enzyme concentration was found at pH 8.0 which is near to pI of Protease A. The increasing of protein solubility decreased with pH brought down to 4.2. Therefore, the result of this experiment was found to follow the theory related to protein solubility. The maximum dry matter percentage was found 20.4% RI that Protease A could hold without any precipitate and haze along with the highest clarity. The optimum protein solubility for Protease A was found with in a conductivity range of 4.0 to 5.0 mS/cm within a pH range of 4.0 to 6.0 with the highest clarity.

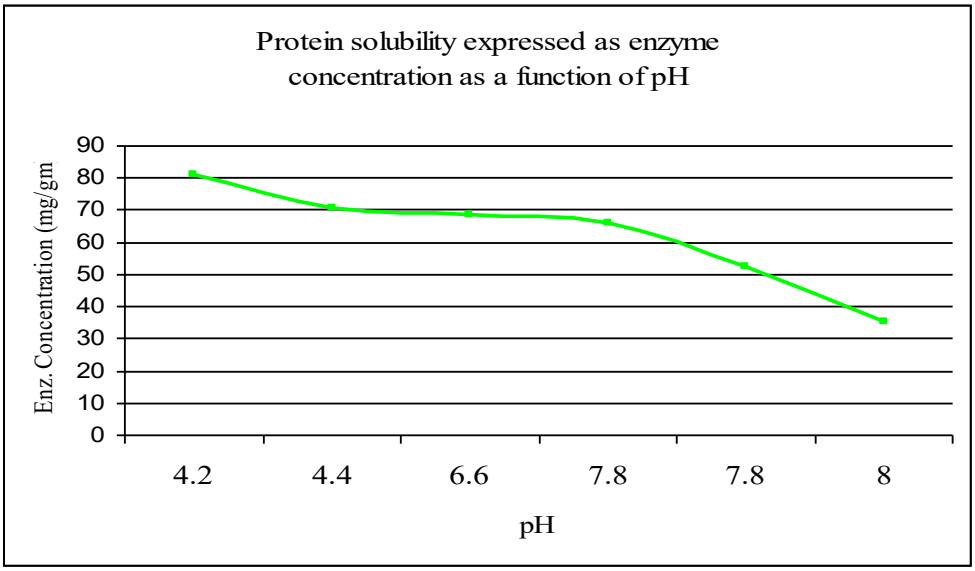

Fig. 1. Protease A solubility region was shown with different enzyme concentration within a pH range of 4.0 to 8.0. Here, protein solubility is expressed as enzyme concentration as a function of pH

Conclusion

The present study demonstrated a complete solubility pattern of Protease A. Generally, factors that usually controls protein solubility e.g., temperature, maturation time and pH were found to have significant effect on the solubility of Protease A. In case of temperature, a longer protein solubility region as a function of both pH and dry matter percentage was found at 4°C for Protease A than that of ambient temperature (Table 1). The maximum dry matter percentage was found 20.4% RI at pH 4.0 that Protease A could hold without any precipitate and haze along with the highest clarity. The optimum protein solubility for Protease A was found within a conductivity range of 4.0 to 5.0 mS/cm and within a pH range of 4.0 to 6.0 with the highest clarity. The observations of the optimization of dry matter percentage, conductivity and pH range on protein solubility suggest that all the enzyme crystals soluble in solution in all the unit operations of downstream processes for Protease A used by Novozymes, Denmark and enzyme loss could be avoided during recovery of industrial enzymes. The solubility region of Protease A with respect to enzyme concentration was found within the range of 81.0 to 35.4 over a wide range of pH starting from 4.0 to 8.0 (Figureure 1). The solubility trend of the protein was found not to be changed by the level of the enzyme concentration. The idea about the Protease A solubility region as a function of pH with respect to enzyme concentration could help to maintain the enzyme concentration in the entire downstream processes for Protease A recovery by Novozymes, Denmark and to keep all the enzyme crystals soluble in solution. This will help to reduce the recovery cost of Novozymes, Denmark which ultimately will reduce the total production cost of Protease A.

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References

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

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

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

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

Faber, C., Hobley, T.J., MOllerup, J., Thomas, O.R.T. and Kaasgaard, S.G. 2007. Factors affecting the solubility of *Bacillus halmapalus* α-amylase. Article in press, *Chemi. Eng. Proces*

Green, A. 1931a. 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

Green, A. 1931b. 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

Green. A.A. 1932. Studies in the physical chemistry of the proteins. *Phys. Chem. Protein.* 10: 47-66

Liu, J.W., Boucher, Y., Stokes, H.W. and 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

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

Retailleau, P., Ries-Kautt, M. and 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

Robert, A., Donahue, Jr. and Bebee, R.L. 1999. BL21-SI competent cells for protein expression in *E. Coli. Focus.* 21: 49-51