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ORIGINAL RESEARCH ARTICLE

Effect of Selected Hofmeister Cations and Anions on Recombinant Protease B Solubility

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ABSTRACT

Cost of the 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. CaCl2 is usually used by Novozymes A/S, Denmark in the pre-treatment of Protease B UF concentrate on early downstream processing. Therefore, it has been tried in these experiments to find out alternative and cheaper cationic salt that could be used in the pre-treatment of Protease B UF concentrate in order to increase protein solubility in a cost-effective strategy of Protease B recovery and production at industrial scale. Beside these, the research work was designed to investigate whether the solubility of Protease B UF concentrate follows the order of the salts in the Hofmeister series against pH or not. Protease B, a recombinant proteolytic enzyme produced from genetically engineered Bacillus licheniformis was used in this study. Six different salts, five different cations (KCl, NaCl, LiCl, MgCl2 and CaCl2) and one anionic salt (Na2SO4) from the Hofmeister series were tested in these experiments. The highest protein solubility in Protease B UF concentrate was found in the presence of MgCl2 at 1.0 M concentration but the most significant and interesting effect on protein solubility was observed by CaCl2. Finally, a qualitative disagreement was observed in the presence of CaCl2.

Keywords: Hofmeister series, Protein solubility, Cationic salt, Anionic salt and Pre-treatment of protein.

INTRODUCTION

Neutral salts varied in their effect on the solubility of proteins stated by Hofmeister in 1988. One group of salts could be ranked according to their efficiency at precipitating proteins, while a second group of salts could be ranked according to their efficiency at solubilizing proteins. Essentially this same total ordering of ions, with the same sign change between the two groups, can be generated by measuring their effect on protein stability or from many different physical measurements of

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aqueous salt solutions, such as the surface potential difference (at an air–water interface), the water activity coefficient, water proton nuclear magnetic longitudinal relaxation rates, infrared spectroscopy, behavior on Sephadex G-10 (gel sieving chromatography), Jones- Dole viscosity B coefficients, and solution neutron diffraction with isotopic substitution. [1-4]

Recently, substantial attention has been paid to Hofmeister phenomena because of their relevance to a broad range of fields. A few examples of physical behavior obeying the Hofmeister series include enzyme activity[5-7], protein stability[8], protein–protein interactions[9, 10], protein crystallization[11], optical rotation of sugar and amino acids[12], as well as bacterial growth.[13] Although Hofmeister effects for macromolecules in aqueous solution are ubiquitous, the molecular-level mechanisms by which ions operate are only beginning to be unraveled.[14]

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.[15-17] 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.[18, 19] A trouble was reported by Novozymes, Denmark in the recovery of Protease B 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 broth by using flocculation, centrifugation or rotary vacuum filtration as a consequent 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 which is determined by various interactions between protein-protein, protein-ion, ion-water, and water-protein molecules.[20] Data on protein solubility first reported in the beginning of the last century.[21] 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.[22, 23] But data on protein solubility as a function of Hofmeister series are limited to a wide range of proteins. Therefore, the present study was aimed to test whether the solubility of Protease B follows the Hofmeister series or not and finally to make a better understanding about the solubility of the enzyme.

MATERIALS AND METHODS

Enzyme

Protease B ultrafiltrate (UF) concentrate (Novozymes A/S, Denmark) was used as the study enzyme 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.

Chemicals

NaOH (Gropa A/S, (Denmark), CH3COOH (Bie & Berntsen, Denmark), 34% CaCl2 (Kemira, Denmark), KCl and Na2SO4 (J.T Baker, The Netherlands), MgCl2.6H20 (Merck, Germany) and CaCl2.2H20 (Sigma–Aldrich, Germany), LiCl and NaCl (Acros, USA) were used in this study. All chemicals used in this study were of analytical grade. Both tap and deionized water was used in the experiments

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 the UF concentrate was diluted two times following the addition of deionised water. A lot of enzyme crystal was seen to be present in an 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 using 0.2 microfilter (Sartorius). The filtrated centrifuged supernatant was used for further experiments as starting material.

Concentrating and Diawash of Protease B UF

After minimal pre-treatment of the Protease B UF, concentrate was further concentrated using a cross flow filtration system (Sartorius Corporation, USA) at ambient temperature up to 23.2% RI 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 23.2% 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. Then the concentrate was defeated with deionised water down to 2.7 ms/cm from 5.0 ms/cm in order to remove salts from the concentrate. The dry matter

percentage of the diafiltered concentrate was then 20.2% RI with 2.7 MS/cm conductivity and 114.9 mg/g enzyme concentration. The diafiltered concentrate was clear with no haze and precipitate was then used as the starting material in the following experiments.

Test of salts in enzyme solutions

A solution of 20 ml diafiltered concentrate (20.2% RI, 2.7 mS/cm conductivity and 114.9 mg/g enzyme concentration) was portioned out into 18 small beakers. A solution of 30 ml deionised water with different amount of each salt was prepared and was then shaked properly to make all the salts completely soluble in deionised water. All salts (KCl, NaCl, LiCl, MgCl2, CaCl2 and Na2SO4) at all molar concentrations were found completely soluble in water except the anionic salt, Na2SO4. Thus, the solution with Na2SO4 was shaked vigorously in order to make the salt completely soluble in water. Finally, the anionic salt (Na2SO4) was found completely soluble in solutions.

A solution of 20 ml diafiltered concentrate was then added into each 30 ml salt solution to make the final volume 50 ml. The final concentration of each salt solution together with UF concentrate was 0.1 M, 0.5 M and 1 M. Thus, 3 beakers of 50 ml solution were prepared with each salt at three different molar concentrations. The enzyme concentration into each 50 ml final solution was 48.94 mg/g enzyme concentration. All the samples were then adjusted to pH 4.0 and were then left at ambient temperature (20-22˚C) and studied again after 12 hours.

After standing for 12 hours at ambient temperature (20-22˚C), all the samples were then mixed homogeneously by rotating on a magnetic stirrer. Both of the sludge and the supernatant were then collected from each samples following centrifugation at 3500 rev/min for 5 minutes. The soluble supernatant was calculated as a percentage (v/v) of every sample by deducing the sludge percentage from 100%. All the supernatant were then sent for enzyme analysis to Enzyme Analysis Laboratory, Novozymes A/S. The sample protein solution without any salt showed no sludge in the centrifuged solution. Following after centrifugation, the soluble supernatant of the sample protein was found 100%. Here, the higher the soluble supernatant fraction corresponds the higher the solubility of an enzyme.

RESULTS AND DISCUSSION

Test of salts in enzyme solution with 48.94 mg/g enzyme concentration

Following after pre-treatment of Protease B UF concentrate as discussed in the materials and methods section was used as the study material in these experiments. Figure 1 and 2 was drawn to show the effect of salt type and salt concentration on protein solubility in terms of soluble supernatant

fraction and enzyme concentration respectively. In the presence of CaCl2 and MgCl2, the fraction of soluble supernatant was found to decrease then it was increased with increased salt concentration starting from 0.5 to 1.0 M. According to the Cohn-Green formula all of these cations showed salting in tendency with increased salt concentration but NaCl behaved as neutral salts in Protease B solubilisation (Figure 1).[21] Both of these cationic and the anion salts behaved as salting out the salt with increased salt concentration (Figure 1).

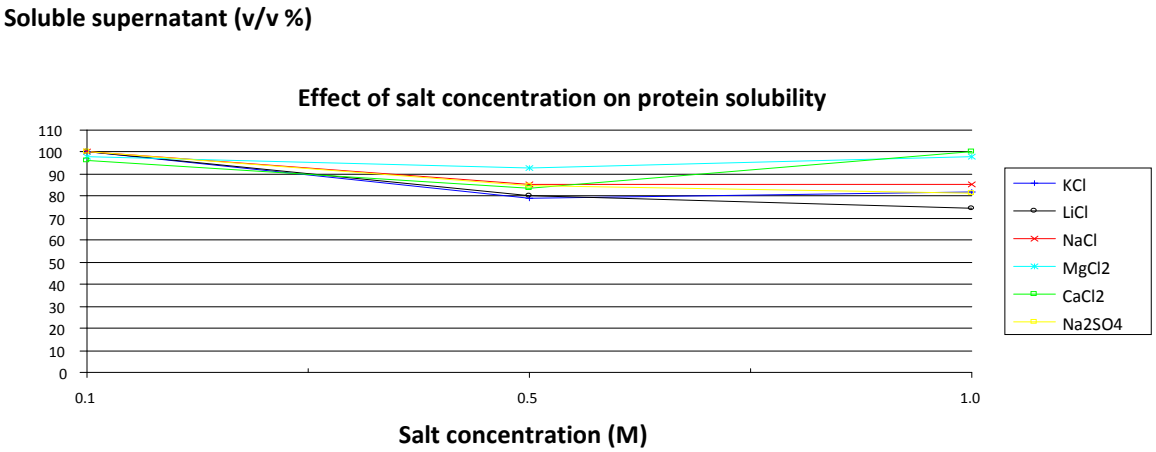

Figure 1: Effect of salt type and salt concentration on protein solubility in terms of enzyme concentration.

In Figure 1, the higher enzyme concentration in the supernatant was shown by MgCl2 but not by CaCl2 though the soluble supernatant fraction of enzyme solutions with both of these two cations were found high. All salts except MgCl2 showed protein solubility with respect to enzyme concentration near to zero at a high salt concentration (1.0 M). The interesting qualitative disagreement between enzyme concentration and soluble supernatant fraction was found in the presence of CaCl2 at different concentrations starting from 0.1 to 1.0 M and thus more experiments were conducted in the presence of CaCl2 in order to find out the actual reason behind this qualitative disagreement. The order of the salts (cation) based on the ability to keep the protein soluble (where the protein solubility is expressed as the soluble supernatant fraction) in supernatant at 1.0 M salt concentration was found as follows.

$$\mathbf{Ca^{2+} > Mg^{2+} > Na^{+} > Li^{+} > K^{+}}$$

Here, the highest protein solubility was found by Ca2+ and the lowest was shown by K+. The order of the cations follows the Hofmeister series except Li+ and Na+. The anion, SO42- shows the most significant affect on protein precipitation and in

opposite shows the least protein solubility as in the Hofmeister series (Figure 1). According to enzyme concentration in the supernatant, the order of the series comes at 1.0 M salt concentration (Figure 2),

$$Mg^{2+} > Na^{+} > Li^{+} > K^{+} > Ca^{2+}$$

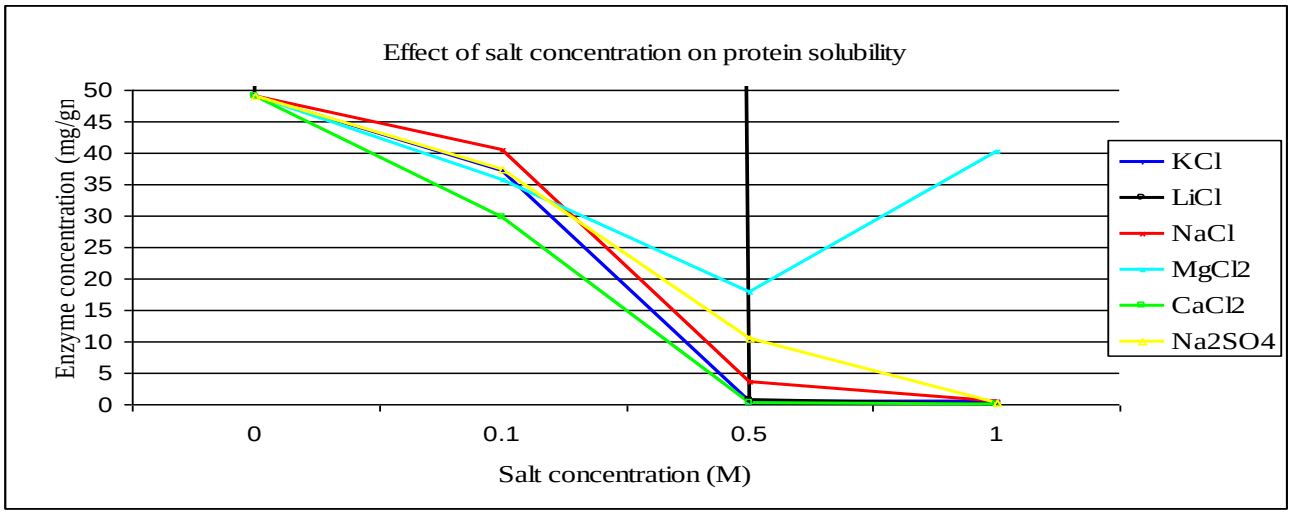

Figure 2: Effect of salt type and salt concentration on protein solubility.

Here, the order of the salts was founded to follow the order as found above (when the solubility is expressed as the soluble supernatant fraction) except Ca2+. Thus, the place of the cation, Ca2+ in the series made based on the ability to keep the protein soluble in enzyme solutions agree the qualitative disagreement in protein solubility as discussed in this experiment.

Test of salts in enzyme solution with lower enzyme concentration (34.47 mg/g)

The experiment was repeated with lower enzyme concentration (34.47 mg/g) with six different salts at three different salt concentrations (0.1 M, 0.5 M and 1.0 M) in order to find out the effect of lower enzyme concentration on protein solubility. The cation NaCl behaved as neutral salt in Protease B solubility (Figure 3). According to Cohn-Green formula salting in was observed by LiCl and KCl with increased salt concentration.[21] While, the anion Na2SO4 and the cation CaCl2 behaved as salting out the salt with increased salt concentration (Figure 2). The highest protein solubility was observed in presence of MgCl2 at 1.0 M salt concentration though the cation behaved as neutral salt.

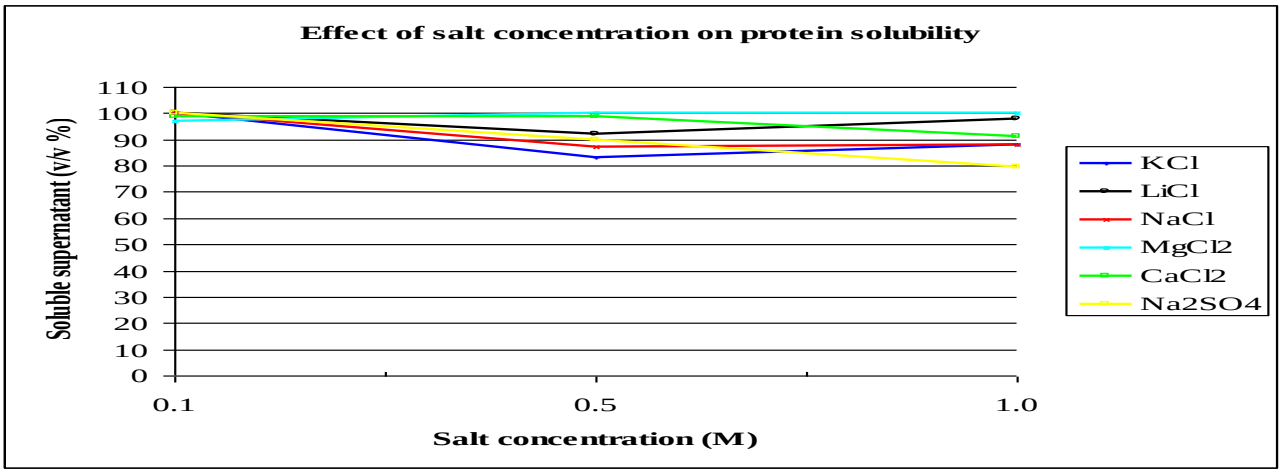

Figure 3: Effect of salt type and salt concentration on protein solubility

The higher the enzyme concentration in supernatant means the higher the rate of enzyme solubility in solutions. The cation MgCl2 showed the highest enzyme solubility with respect to enzyme concentration and soluble supernatant fraction than that of others and behaved as neutral salt. The anionic salt, Na2SO4 was found to show salting out tendency. The cations, LiCl and NaCl were found to behave as salting in salt. While, the cations, KCl and CaCl2 were found to behave as neutral salts.

The order of the series of salts at 1.0 M salt concentration was found as follows based on the ability to keep the protein soluble in solutions when the solubility is expressed as the fraction of the soluble supernatant.

$$\mathbf{Mg^{2+} > Li^{+} > Ca^{2+} > K^{+} \approx Na^{+}}$$

Here, the order of the salts was founded to follow the order as found above (when the solubility is expressed as the soluble supernatant fraction) except Ca2+. Thus, the place of the cation, Ca2+ in the series made based on the ability to keep the protein soluble in enzyme solutions agree the qualitative disagreement in protein solubility as discussed in this experiment. And the effect of divalent cation in comparing to monovalent Keaton was found more significant on protein solubility.

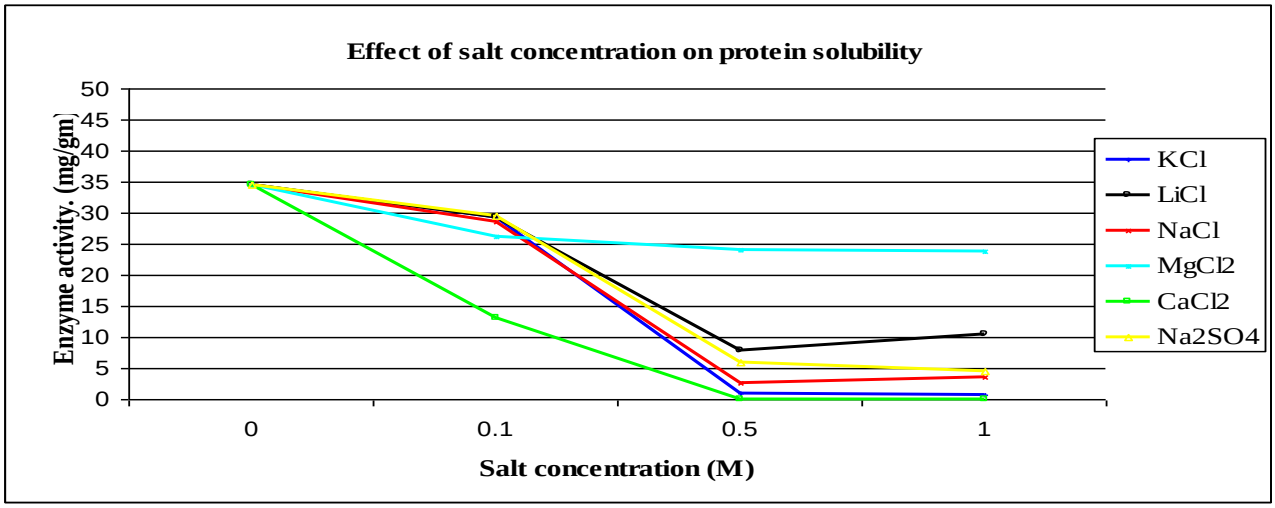

Figure 4: Effect of salt type and salt concentration on protein solubility

Figure 4 showed the same result depending on the enzyme concentration as was found in figure 25 except CaCl2. In figure 24, the highest enzyme concentration in the supernatant was shown by MgCl2. Thus, the highest protein solubility was shown by MgCl2. A little bit decreased soluble supernatant fraction was found in the presence of CaCl2. But the lowest enzyme concentration by CaCl2 was found in the supernatant. Thus, the same qualitative disagreement was shown in this experiment by CaCl2. Therefore, more experiments were needed to perform in the presence of CaCl2 in order to find out the actual reason behind this qualitative disagreement. The protein solubility was found to decrease first with increased salt concentration starting from 0.1 to 0.5 M and then the solubility was found to either increase or constant depending on the cations. In conclusion, the trend of the Protease B solubility was not affected by the concentration of the protein.

Test of CaCl2 in enzyme solution with higher enzyme concentration (48.94 mg/g)

Two more experiments were done with different CaCl2 concentration starting from 0.1 to 1.0 M to check the result that was found from both of the previous experiments and to find out the complete solubility behavior of Protease B UF concentrate in present of CaCl2. Salting in was shown by CaCl2 in higher enzyme concentration while in lower enzyme concentration salting out was obtained at the highest salt concentration (1.0 M). Thus, two more experiments were conducted in order to find out, is the result reliable or not? The result obtained from both of these two experiments was found interesting in the presence of CaCl2. At higher salt concentration (1.0 M) the soluble supernatant fraction was found 100% which means the percentage of original enzyme concentration in supernatant should be higher as in case of MgCl2

(Figure 1 and 2) Thus, the new experiment was performed to make a more detailed study of CaCl2 on Protease B UF concentrate and solubility behavior. All the data obtained from this experiment is given in the following tables (Table 1 and 2). In the second experiment, the new idea was come that all the samples those were left in the freezer (in order to send them later for enzyme concentration analysis to an Enzyme analysis laboratory, Novozymes, Denmark) were towed back after 12 hours to solutions in order to investigate whether the enzyme was found to come back with solutions or not. After thawing at ambient temperature, the precipitate was found in all enzyme samples except the sample with 0.1 M salt concentration. Precipitate in all enzyme samples was then calculated as a percentage and is given in the table 2. After that all the samples along with precipitate was shaken in order to solubilize protein and was found soluble but all the samples were not founded completely clear. Then, all the samples were then stored in the freezer and send those again for enzyme analysis.

Table 1: Effect of different CaCl2 concentrations of Protease B filtered supernatant solubility at pH 4.0 at ambient temperature after 12 hours.

Type of salt Solutions	Final solution concentration (M)	Soluble supernatant fractio (v/v %)	Enzyme concentration in supernatant (mg/g)
$CaCl_2.2H_20$	0.1	91.0	7.35
$CaCl_2.2H_20$	0.2	83.0	<0.03
$CaCl_2.2H_20$	0.3	83.0	<0.03
$CaCl_2.2H_20$	0.4	85.5	<0.03
$CaCl_2.2H_20$	0.5	84.5	<0.03
$CaCl_2.2H_20$	0.6	86.5	<0.03
$CaCl_2.2H_20$	0.7	90.0	<0.03
$CaCl_2.2H_20$	0.8	95.5	0.06
$CaCl_2.2H_20$	0.9	100.0	0.05

$CaCl_2.2H_20$	1.0	100.0	0.07

Table 2: Effect of different CaCl2 concentrations on Protease filteresupernatant solubility at pH 4.0 at ambient temperature after 12 hours.

Type of salt Solutions	Final solution concentratio (M)	Soluble supernatant fraction (v/v %)	Precipitate after thawing supernatant at ambient temp. After 12 hrs (% v/v)	Enzyme concentration in supernatant (mg/g)
$CaCl_2.2H_20$	0.1	93.0	0.0	9.33
$CaCl_2.2H_20$	0.2	84.0	2.3	<0.03
$CaCl_2.2H_20$	0.3	83.5	2.9	<0.03
$CaCl_2.2H_20$	0.4	84.5	2.3	<0.03
$CaCl_2.2H_20$	0.5	85.0	2.8	<0.03
$CaCl_2.2H_20$	0.6	85.5	6.0	<0.03
$CaCl_2.2H_20$	0.7	88.0	5.9	<0.03
$CaCl_2.2H_20$	0.8	94.5	9.3	0.04
$CaCl_2.2H_20$	0.9	97.0	9.7	0.04
$CaCl_2.2H_20$	1.0	99.0	9.8	0.05

The result of both of the experiments shows that the soluble supernatant fraction is decreasing with increased salt concentration though the enzyme concentration in supernatant is not increasing with increased salt concentration up to 0.5 M. But, the percentage of supernatant was found to increase with a little increase of percentage of original enzyme concentration in the supernatant with increased salt concentration starting from 0.5 to 1.0 M. As the enzyme concentration is qualitatively correlated to the percentage of supernatant as a factor of protein solubility, the percentage of enzyme concentration in supernatant should be higher than that was found in these experiments. Thus, the result of both of these experiments showed that something wrong in the presence of CaCl2 with respect to enzyme concentration in terms of protein solubility. Thus, the supernatant was thawed again before sending for enzyme analysis and a lot of enzymes were found as precipitate at the bottom of all the test tubes that could be the reason of the dramatic loss of the enzyme concentration in the supernatant. All the samples were thawed at ambient temperature. Therefore, temperature was found to have an effect on protein solubility. The idea comes from this experiment that all the enzyme samples should send for enzyme analysis immediately following just after the experiment and should not stored in freezer before sending to Enzyme analysis laboratory. Salting in was shown by CaCl2 at the highest salt concentration (1.0 M) in both experiments with higher enzyme concentration when the solubility is expressed as the percentage of the original enzyme concentration in the supernatant. Thus, the result was obtained from the experiment in higher enzyme concentration was reliable.

Test of MgCl2 in enzyme solution with higher enzyme concentration (48.94 mg/g)

The following experiment was performed in order to find out the complete solubility behavior of Protease B concentrate (48.94 mg/g enzyme concentration) in the presence of MgCl2 of different concentrations starting from 0.1 to 1.0 M as a function of enzyme concentration and percentage of supernatant. Figure 5 was drawn using the data obtained from this experiment.

The result of this experiment showed that MgCl2 behaved as salting in cationic salt (Figure 5) as was found in the first experiment with higher enzyme concentration. The percentage of supernatant was increasing with increased salt concentration starting from 0.4 M to 1.0 M, the percentage of original enzyme concentration in the supernatant was found

to fluctuate. But at 1.0 M salt concentration, the enzyme concentration was increased sharply to 34.01. Thus, the solubility pattern of Protease B concentrates with respect to enzyme concentration and soluble supernatant fraction was found same at highest (1.0 M) salt concentration but not found a qualitatively correlation between enzyme concentration and the soluble supernatant fraction.

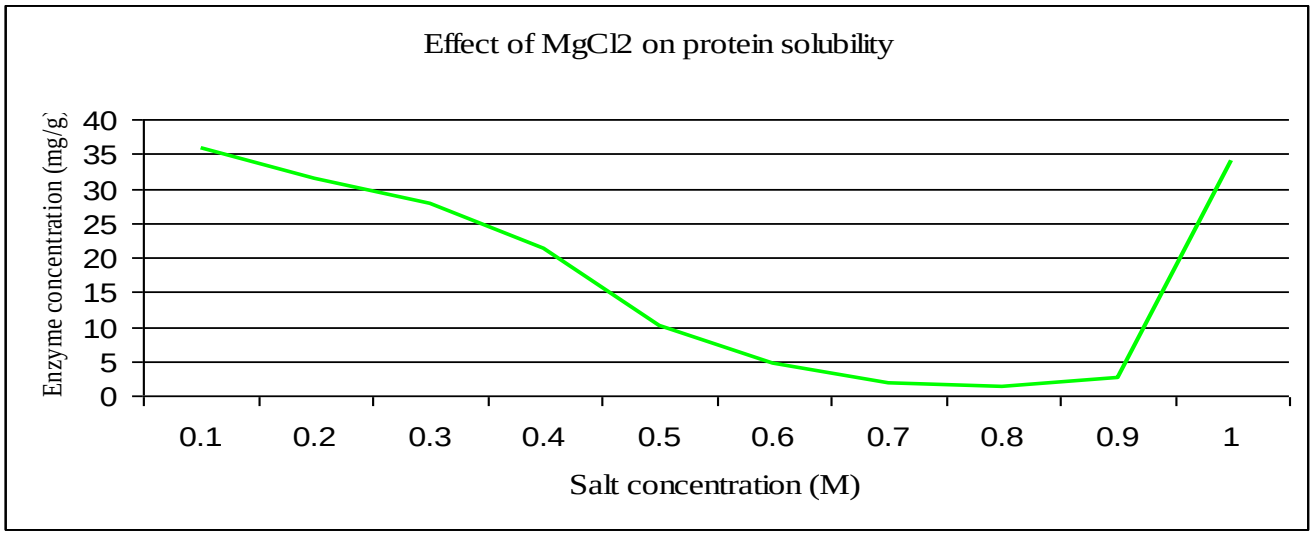

Figure 5: Effect of MgCl2 on protein solubility

Test of mixed salts (CaCl2 and MgCl2) in enzyme solution with higher enzyme concentration (48.94 mg/g)

The following experiment was conducted with Protease B UF concentrate (48.94 mg/g enzyme concentration) in the presence of mixed salts (CaCl2 and MgCl2) at different concentrations starting from 0.1 to 1.0 M in order to find out the solubility behavior of Protease B. All the samples with different salt concentrations were left for 12 hours at ambient temperature. Volume of supernatant of all samples was calculated as a percentage. All the supernatant were then stored in freezer before sending for enzyme analysis. As the relation between the percentage of supernatant and percentage of enzyme concentration in terms of protein solubility was found non correlated, the supernatant was thawed again at ambient temperature before sending for enzyme analysis in order to check whether the supernatant become completely soluble or not. After thawing, a lot of enzyme of most of the samples was found to keep the place as precipitate in the bottom of the test tubes. Then the precipitate of all samples was calculated as a volumetric percentage. All the data

obtained from this experiment is used to draw the Figure 6.

The result of this experiment showed that the high protein solubility at 0.1 M salt concentration as a function of the soluble supernatant fraction and the enzyme concentration (Figure 6) and the volumetric percentage of enzyme precipitate after the thawing of the supernatant after 12 hours at ambient temperature was found zero which shows a qualitative and positive correlation between each other in terms of protein solubility. The soluble supernatant fraction was found to be a little bit fluctuated at different salt concentrations (from 0.2 to 0.8 M) with a sharp decrease in enzyme concentration in supernatant showing salting in (at 1.0 M salt concentration) and a non qualitative correlation between each other. But a lot of enzymes were found to be precipitated at the bottom of the test tubes. Which could be the reason for dramatic loss of the enzyme concentration in the supernatant though the percentage of supernatant was high at different salt concentrations. Finally, at 1.0 M concentration the enzyme concentration was found to regain at expected levels.

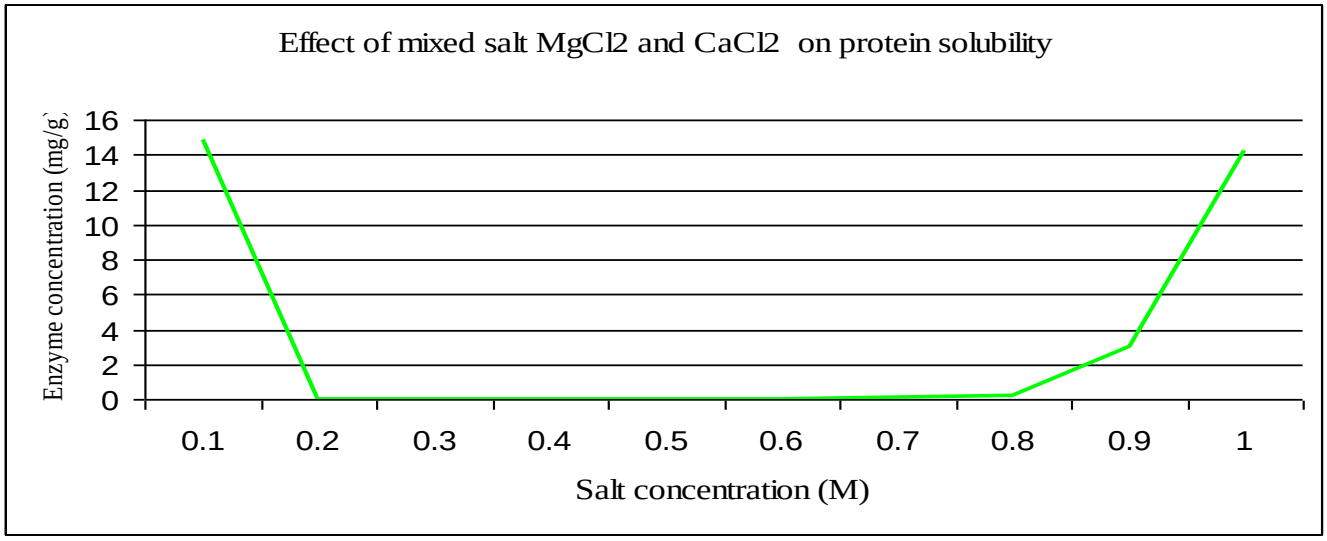

Figure 6: Effect of mixed salt (MgCl2 and CaCl2) on protein solubility.

Test of CaCl2 (salt concentration, 0.01 to 0.10 M) in enzyme solution with higher enzyme concentration (48.94 mg/g)

The experiment with higher enzyme concentration was repeated with Protease B UF concentrate at different CaCl2 concentration starting from 0.01 to 0.1 M in order to find the solubility pattern of

Protease B UF concentrate at lower salt concentrations. All the data obtained from this experiment is given in the following table (Table 3).

At lower salt concentrations, a little bit salting out tendency (According to Cohn-Green formula) was observed by CaCl2 because the solubility (as a function of soluble supernatant fraction) of Protease B was found to increase first up to 0.05 M salt concentration, then it was found to decrease up to 0.1 M salt concentration.[21] Both of the soluble supernatant fraction and the percentage of the original enzyme concentration in supernatant were found at an expected level. Thus, a positive qualitative correlation between the percentage of soluble supernatant and the percentage of the original enzyme concentration in supernatant was found and no precipitate after thawing supernatant at room temperature after 12 hours was noted at lower salt concentrations. The In conclusion, the higher concentration of CaCl2 was found to have significant effect on protein solubility instead of lower CaCl2 concentration.

CONCLUSION

The highest protein solubility in Protease B UF concentrate was found in the presence of MgCl2 at 1.0 M concentration but the most significant and interesting effect on protein solubility was observed by CaCl2. Thus, a couple of experiments were conducted in the presence of both MgCl2 and CaCl2. Enzyme solubility for Protease B was expressed as volumetric soluble supernatant fraction and enzyme concentration in the supernatant. A qualitative disagreement was observed in the presence of CaCl2. The reason behind this qualitative disagreement was found that the centrifuged supernatant was found to form enzyme precipitate when the supernatant was towed back into the form of solutions. Thus, the important message from this research was found that the enzyme samples (supernatant) with CaCl2 should not keep in freezer before sending for enzyme analysis.

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