Study of the Solubility of a Modified *Bacillus licheniformis* α-Amylase around the Isoelectric Point

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The solubility of a modified recombinant *Bacillus licheniformis* α-amylase (mBLA) has been studied by batch crystallization. A semi-pure preparation was chosen containing five isoforms with pI values from 6 to 7.3 (weighted average of 6.6). Small amounts (<1 %) of protein impurities were also present. Solubility was studied in the pH range of 6 to 8. The lowest solubility without added salts was 60 mg·mL^{-1} at pH 7. The addition of 0.1 mol·L^{-1} sodium salts of nitrate, sulfate, and thiocyanate had a small effect on solubility. However, solubility was lowered significantly by adding 0.5 mol·L^{-1} sodium sulfate at all pH values and increased with 0.5 mol·L^{-1} sodium thiocyanate at pH 7 and pH 8. The effect of anions on α-amylase solubility followed the Hofmeister series, and only weak evidence of reversal was seen below the isoelectric point. Cations had little effect on solubility. The sign and magnitude of the α-amylase ζ potential was determined in the presence and absence of 0.1 mol·L^{-1} salt. Qualitatively, ζ potential correctly predicted the different salts influence on mBLA solubility.

Introduction

Due to the demands for new types of highly concentrated protein formulations of industrial enzymes as well as alternative methods of delivery within the pharmaceutical industry, interest in solubility and bulk crystallization of proteins has recently increased.[1] The rapid development of scalable, reproducible, and robust crystallization processes in product recovery and formulation leading to competitive products requires detailed knowledge of the solid−liquid equilibrium[2] together with information on how various precipitants such as salts affect the solubility properties of a given protein, particularly when operating at or above its solubility limit. Comprehensive solubility data are available for only very few proteins, probably the best example being highly pure lysozyme. Furthermore, the number of studies published on bulk crystallization in the presence of impurities is very low. Indeed, the crystallization of ovalbumin[3] and nucleation and growth of microbial lipase crystals[4] represent two of the very few examples. In contrast, the influence of salts on the solubility of highly purified proteins (such as lysozyme, collagenase, and carboxyhemoglobin) and the bovine pancreatic trypsin inhibitor (BPTI) has been intensively studied.[5−12] The strong impact of salts on a protein's solubility has been demonstrated in all cases, and a characteristic order of efficiency has been found in which the salts (i.e., cations and anions) precipitate the protein out of solution. This order of efficiency is commonly referred to as the Hofmeister series[5] and has been confirmed for many but not all proteins (e.g., reversal of the anion series has been observed for lysozyme).[9] Riès-Kautt and Ducruix[9] argue that the order of this series is dependent on the sign of the protein's net charge and thus the pH of the solution should be shifted from values less than to values more than the isoelectric point (pI) of the protein or vice versa. A reversal of the order of efficiency in which anions influence the solubility depending on the protein's net charge has only been shown for fibrinogen,[13] and while this reversal should not occur for cations,[14] this has never been systematically verified.

From a process point of view, an inverse of the effects of different salts on solubility depending on the sign of a protein's net charge would be of significant importance in the case where salts are used to steer the protein's solubility. Accordingly, the aim of this study has been to further the understanding of how salts and pH influence the solubility of proteins. We wished to evaluate whether the conclusions made by others for highly purified protein systems (e.g., lysozyme) can be transferred to the more heterogeneous protein solution studied here. A modified *B. licheniformis* α-amylase has been used, and although the absolute solubilities at different conditions naturally will depend on the amino acid sequence and folding of the protein, the main objective of this paper is to identify some common rules for how pH and salts influence the crystallizability of proteins. Of particular interest to us was how anions and cations influence the solubility of a protein depending on the sign of the net charge, and measurements were thus made just below the isoelectric point of the amylase studied. Evaluation of ζ potential measurements for estimating the sign and magnitude of the net charge in impure protein solutions coupled with its utility in aiding rapid identification of conditions of low and high solubility was a second objective of this study.

Experimental Section

Preparation of Bacillus licheniformis* α-*Amylase. A modified, recombinant *Bacillus licheniformis* α-amylase was employed in this work, batch number PDS-2003-00337. The exact details are confidential. However, one methionine has been

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replaced, and the N-terminal of the protein has been modified. The α-amylase was expressed in a recombinant *Bacillus licheniformis*. It was fermented and purified at Novozymes A/S, Bagsværd, Denmark. Cells and other debris were removed by filtration. The pH of the filtrate, containing the α-amylase, was adjusted to pH 10.5 and concentrated 10-fold by ultrafiltration (10 kDa cutoff) at 10 °C. The enzyme concentrate was crystallized by lowering the pH to 7.5, and the crystals were subsequently harvested by centrifugation. The crystal cake was thoroughly washed by suspending twice in water followed by centrifugation, and this preparation formed the initial feedstock employed in this study.

Reagents. Sodium nitrate was obtained from Merck (Darmstadt, Germany). Sodium sulfate and calcium chloride were purchased from J. T. Baker (Deventer, The Netherlands). Sodium thiocyanate, lithium nitrate, and cesium nitrate were supplied by Sigma-Aldrich (Steinheim, Germany). The buffers 2-(*N*-morpholino)ethanesulfonic acid (MES) and *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES) were also obtained from Sigma-Aldrich, whereas boric acid was supplied by AppliChem (Darmstadt, Germany). Sodium hydroxide and acetic acid (at concentrations of 0.1 and 1 mol·L^{-1}, respectively) were provided by Bie & Berntsen (Rødovre, Denmark). All chemicals were of analytical grade.

Crystallization Studies. All crystallization processes were conducted using a buffer composed of a mixture of 10 mmol·L^{-1} MES, 10 mmol·L^{-1} HEPES, and 10 mmol·L^{-1} boric acid adjusted to the desired pH by adding sodium hydroxide. All samples were prepared in ultrapure water of 18.2 MΩ·cm^{-1} conductivity (Millipore, Billerica, MA), together with 0.2 % (w·v^{-1}) of the antimicrobial agent Proxel LV (Avecia, Manchester, UK) to prevent any interference from microbial contamination.

Depending on the pH value of interest, crystals of α-amylase were either (i) dissolved in the buffer at pH 9.5 and then filtered through a 0.22 μm pore size cellulose acetate filter (Sartorius, Göttingen, Germany) or (ii) suspended at pH 5.5 and stirred until they dissolved; subsequently, more crystals were added until no further material could be dissolved. The resulting crystal slurry was centrifuged, and the supernatant was filtered through a depth filter (Seitz, Bad Kreuznach, Germany) of 0.22 μm pore size. The crystallization experiments were conducted in batch mode. For every condition tested, solutions of two different initial protein concentrations were prepared in the buffer as specified above, containing the precipitants of interest (i.e., salts) as required. With the exception of lithium nitrate, which was added in liquid form as a 3 mol·L^{-1} stock solution prepared in the buffer described above, all other salts were added in solid form. For each preparation, the crystallization was initialized by slowly (over 300 s) raising or reducing the pH to the value of interest, through the addition of sodium hydroxide or acetic acid, respectively. Aliquots (750 μL) of the solution were then transferred into a series of 1.5 mL Eppendorf tubes before placing in a thermomixer (model 5355, Eppendorf, Hamburg, Germany), which ensured precise temperature control (40 ± 1 °C) and appropriate mixing (1400 rpm). On the basis of visual inspection, it was apparent that no crystals or precipitates formed during pH adjustment and liquid handling. After 96 h of incubation, the tubes were removed from the mixer, and the precipitates formed were analyzed by light microscopy to determine whether they were amorphous or crystalline. In cases where the precipitate was found to be amorphous, the experiment was repeated using a lower initial protein concentration. Crystals were separated from the liquid by centrifugation at 25000g_{av} for 120 s in a temperature-controlled microcentrifuge (model 5415 R, Eppendorf) operated at 40 °C. The supernatants were collected, filtered through 0.22 μm filters (Sartorius), and kept at −30 °C until required for analysis. Upon thawing, no crystals were observed in any samples. The pH was checked at the end of a given crystallization process, and no adjustments were made during an experiment. Deviations from the desired pH value did not exceed ± 0.2 pH units. In a separate experiment, the supernatant protein concentration was determined at different experimental times. For all tested conditions, the protein concentrations in the supernatant did not change significantly (i.e., < 3 %) between 72 h and 96 h, indicating that the equilibrium concentration had been approached within the experimental time employed in the current study.

Analysis. The concentration of total protein was measured using a Cobas Fara spectrophotometric robot (Roche, Rotkreutz, Switzerland) with the ESL assay (Roche, Mannheim, Germany), which is based on a reverse biuret method combined with a copper−bathcuproine chelate reaction.[15] The Cobas Fara was programmed to preheat the reagents and samples for 600 s at 25 °C, then mix them, and 30 s later measure the absorbance at 485 nm. Protein concentrations were determined from a standard curve constructed using bovine serum albumin (BSA) and are expressed in BSA equivalents. The salts employed in crystallization studies were found not to interfere in the assay.

The crystals in the feedstock were re-dissolved and checked for purity by reducing SDS−PAGE (NuPage 10 % Bis−Tris gel NP0301, Invitrogen, Carlsbad, CA). A standard mixture of proteins of known molecular weight (LMW 17-0446-01, GE Healthcare, Uppsala, Sweden) was used to characterize the proteins encountered in the bands. Isoelectric points of mBLA were determined by isoelectric focusing (IEF) in 10-well Novex gels with a pH range of 3 to 10 (Invitrogen), and the gel was calibrated using a commercially obtained protein mixture (SERVA liquid mix IEF markers pH 3 to 10, Invitrogen). IEF runs were conducted in an XCell Surelock Mini-Cell using Novex IEF anode and cathode buffers of pH 3 to 10 (Invitrogen) according to the manufacturer's instructions. All gels from SDS-PAGE and IEF runs were stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich, Steinheim, Germany). Relative estimates of contaminating proteins and isoform compositions in SDS−PAGE and IEF gels, respectively, were obtained by scanning densitometry using the gel analysis software Quantity One (Bio-Rad, Hercules, CA).

Amylase activity in the IEF gels was confirmed in the following way. Bands were cut from an unstained gel, using a stained gel as template. To extract the enzyme from the gel fragments, these were placed in 600 μL Eppendorf tubes that had been pierced at their base with a fine gauge syringe needle. These tubes were then placed in 1.5 mL Eppendorf tubes and spun in a microfuge, which extruded the gel fragments into a slurry giving efficient homogenization. The homogenized gel slurries now contained in the 1.5 mL Eppendorf tubes were resuspended with 1 mL of 15 mmol·L^{-1} $CaCl_2$ to extract the protein. After incubation for 1 h, the samples were recentrifuged to remove the extracted gel pieces. The presence of amylase activity in the supernatants was qualitatively demonstrated using the Phadebas kit (product no. 10-5380-33, GE Healthcare).

The supernatant obtained from batch crystallization experiments was diluted 10-fold using buffer of the same composition (i.e., with respect to salt type, concentration, and pH) as that of the samples. The samples were filtered through 0.22 μm filters (Sartorius), and the ζ potential of each was measured in a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) using

folded capillary cells of 0.75 mL sample volume (Malvern Instruments). Phase analysis light scattering (PALS) was employed to determine the electrophoretic mobility from which the ζ potential was calculated using the Smoluchowski approximation. As the protein and salt concentrations were low (i.e., < 10 mg·mL^{-1} and 0.1 mol·L^{-1}, respectively), the viscosity of the samples was assumed to be equal to that of water. Measurements were conducted in triplicates, each of which consisted of 30 individual measurements. The standard deviation of the mean ζ potential values was in the range of ± 1 mV. The ζ potential was shown to be independent of the dilutions employed in the current study, as has also been reported for other systems (e.g., subtilisin).[16] The ζ potential measurements were only conducted at 0.1 mol·L^{-1} salt concentrations; at 0.5 mol·L^{-1} the data quality deteriorated significantly, and reproducible values of the ζ potential could not be obtained. No general rules as to the maximum salt concentration which permit useful determinations of the ζ potential to be made can be given and should be determined carefully for each system and device being tested. Successful measurements of the ζ potential of proteins (e.g., bovine serum albumin at 0.1 mol·L^{-1} sodium chloride,[17] *Bacillus halmapalus* α-amylase at a conductivity of 25 mS·cm^{-1}), intact and disrupted *Escherichia coli*, and yeast cells at 32 mS·cm^{-1} conductivity[18] conducted with similar equipment have been reported previously. Measurement of the ζ potential of lysozyme crystals at a conductivity as high as 50 mS·cm^{-1} using monovalent electrolytes has been performed with different instruments. In these studies problems were encountered at certain pH values, and additionally, measurements in divalent electrolytes (i.e., either divalent cations or anions) could not made due to low electric fields.[19] The sample conductivity was always less than 16 mS·cm^{-1} during ζ potential measurements.

Results and Discussion

Properties of the Feedstock. We have used a modified α-amylase from *B. licheniformis*, which has had a methionine substituted and the N-terminal modified, thus the properties of the feedstock and protein were first examined. The purity of the feedstock containing mBLA was examined by SDS−PAGE. One major band with a size of 55 kDa was observed in Coomassie Blue stained gels, consistent with the molecular weight of the amylase (Figure 1A). A number of species with lower molecular weights were also detected, but these accounted for less than 1 % of the total protein as determined by gel densitometry. Isoelectric focusing followed by Coomassie Blue staining revealed five bands on the gels with pI values ranging from 6.0 to 7.3 (Figure 1B). The most abundant isoform (pI 6.8) accounted for 30 % of the total protein (determined by gel densitometry), and the least abundant (pI 6) accounted for only 10 % of the total protein. Interestingly, three isoforms were found in very close proximity to each other in the IEF gels, running at pH 6, 6.15, and 6.3. Collectively, these three species accounted for 45 % of the total protein. Amylase activity was associated with all five Coomassie Blue stained isoforms (i.e., detected in all five gel slice fragments), although it is not known how they differ from one another. All isoforms ran as a 55 kDa species in SDS−PAGE, and it is known that the production organism only contained a single gene encoding the α-amylase. Thus, given that all isoforms exhibited α-amylase activity, it is probable that differences in their pI may be due to the modification of certain amino acid side chains (e.g., deamidation). Solutions of mBLA exhibited a brownish color, possibly caused by the presence of non-proteinaceous impurities (e.g., small amounts of carbohydrates and salts). The same pI profile

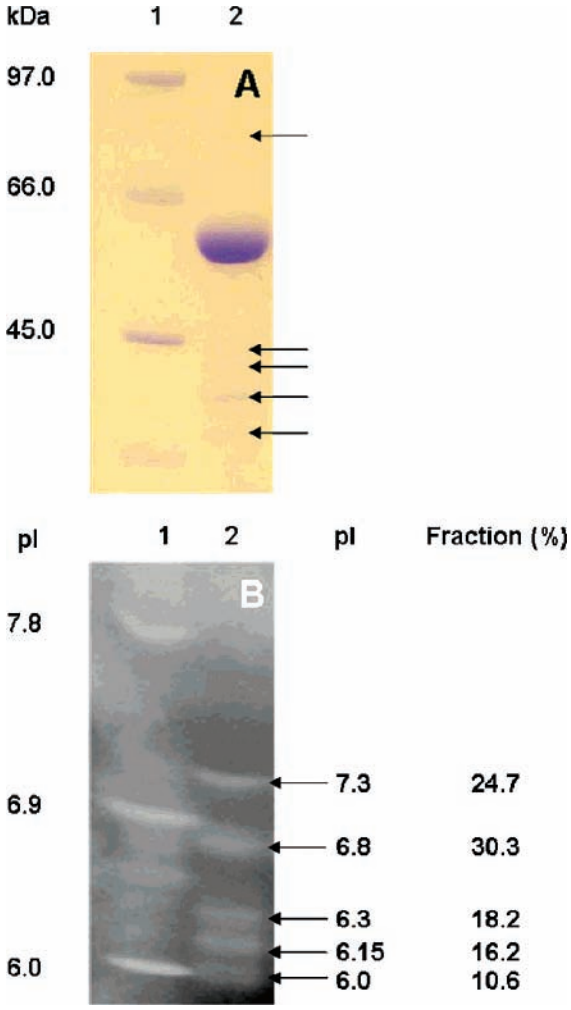

Figure 1. Examination of the modified, recombinant *B. licheniformis* α-amylase feedstock. (A) SDS−PAGE analysis. The molecular weight standards are shown in lane 1, and the arrows show the position of the bands stemming from contaminating proteins. (B) Isoelectric focusing. The calibration standards are in lane 1. The arrows show the position of the isoforms.

was obtained after a recrystallization of the feedstock, suggesting that no discrimination between the isoforms took place during crystallization (data not shown). Although the disturbing influence of contaminants on crystallization processes has been demonstrated previously,[20] the enzyme employed in this study was found to crystallize readily on either side of the pI range found, forming thin, rhombus-shaped crystals. When measured in the buffer specified above, in the absence of salts the solubility of mBLA solutions was lowest at pH 7 with 60 mg·mL^{-1} protein remaining in solution. At pH 6, the solubility doubled to 125 mg·mL^{-1}, and at pH 8, approximately 85 mg·mL^{-1} of mBLA could be solubilized (Figure 2A). It is well-known that the solubility of proteins reaches a minimum at the pI of the protein at low salt concentrations. The U-formed shape of the solubility curve in Figure 2 suggests that even though the amylase consists of a mixed population having different pI values, they behave as a homogeneous population with a pI close to the weighed average pI of 6.6 of the mixed population.

Solubility as a Function of Salt Concentration. (i) Effect of Anions on Solubility of mBLA. The effects of the sodium salts of nitrate, sulfate, and thiocyanate employed at two different concentrations (i.e., 0.1 and 0.5 mol·L^{-1}) and at three different pH values (6.0, 7.0, and 8.1) spanning the pI of mBLA were examined (Figure 2, panels B and C, respectively). At the

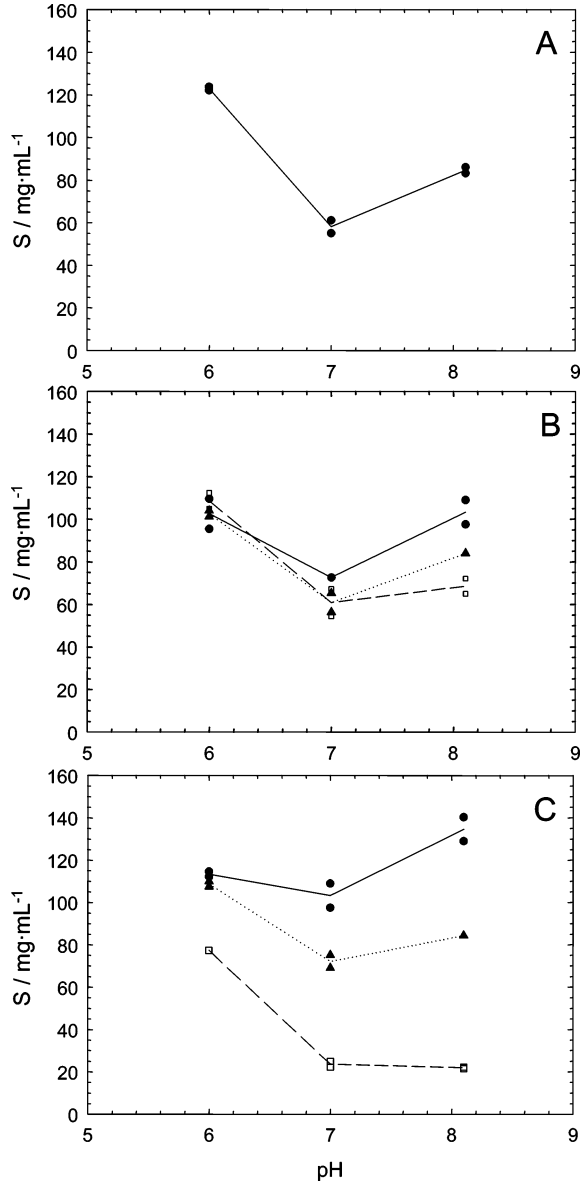

Figure 2. mBLA solubility *S* as a function of pH or added salts. The crystallization process was conducted in batch mode started from two different initial supersaturations in a MES, HEPES, and boric acid buffer (containing 10 mmol·L^{-1} of each). The experiments were conducted at 40 °C for 96 h. (A) mBLA as a function of pH without salt added. (B and C) Influence of anions (0.1 mol·L^{-1} and 0.5 mol·L^{-1}, respectively) on mBLA solubility as a function of pH: ●, influence of sodium thiocyanate; □, sodium sulfate; and ▲, sodium nitrate. All measurements at 40 °C after 96 h.

0.1 mol·L^{-1} concentration level studied, the sulfate anion resulted in the lowest solubility, followed by nitrate and then thiocyanate at pH 8.1, consistent with the Hofmeister series. The effects of the anions at pH 7 were less pronounced. At pH 6, the series seemed to be reversed, although the differences between the effects of the salts are very small. At the higher salt concentrations the Hofmeister series appeared to be preserved at all pH values studied (i.e., at the pI and on either side of it; Figure 2C). In the presence of the salts tested at both concentrations, the solubility at pH 6 was 10 % to 20 % lower than without added salts (see Figure 2A), with 0.5 mol·L^{-1} sulfate reducing the solubility by approximately 35 % (Figure 2 C). Interestingly, at pH 7 the solubility was increased in the presence of 0.5 mol·L^{-1} nitrate and particularly 0.5 mol·L^{-1} thiocyanate (cf., the salt-free case; Figure 2A), whereas 0.5

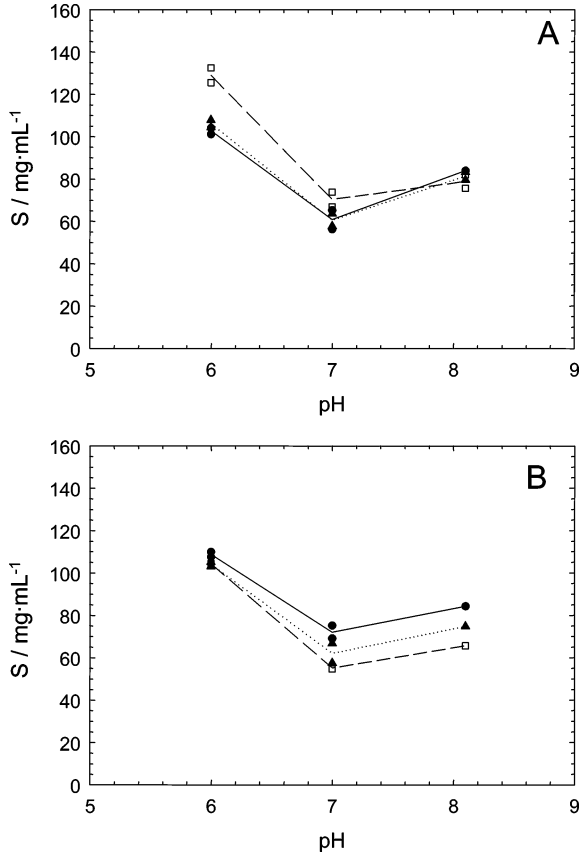

Figure 3. Influence of cations on mBLA solubility *S* as a function of pH: ●, influence of sodium nitrate; □, lithium nitrate; and ▲, cesium nitrate. All measurements at 40 °C after 96 h. Two different initial supersaturations buffered in MES, HEPES, and boric acid (10 mmol·L^{-1} of each) were used. Salt concentrations of (A) 0.1 mol·L^{-1} and (B) 0.5 mol·L^{-1} were employed.

mol·L^{-1} sulfate lowered the solubility drastically (Figure 2C). These trends were also observed at pH 8 in the presence of 0.5 mol·L^{-1} of each salt and to a lesser extent with 0.1 mol·L^{-1} salt.

(ii) Effects of Cations on Solubility. The results presented in Figure 3A,B show that the effects of the cations on solubility were much less dramatic than was observed for the anions. In all cases, the solubility of mBLA solution was lowest at pH 7 and highest at pH 6. When 0.1 mol·L^{-1} lithium salt was used, essentially no effect on the solubility at pH 6 was seen as compared to the case without any added salt, whereas for cesium and sodium nitrates a 20 % reduction at pH 6 occurred. At pH 7, 0.1 mol·L^{-1} lithium nitrate increased the solubility whereas 0.1 mol·L^{-1} levels of the other two salts did not impose any notable effects cf. the salt-free control case (Figure 2A). At pH 8, the addition of the three different nitrate salts at a concentration of 0.1 mol·L^{-1} exerted little effect on the solubility of mBLA (Figure 3A) but suggested a weak tendency toward a reversal of the series, although the differences at pH 8 are within experimental error. When salts were added at 0.5 mol·L^{-1} (Figure 3B), different effects were noted. For example, the lowest solubility was observed with lithium followed by cesium and then sodium. This sequence does not concur with other reports dealing with the influence of cations on solubility. For example, in the case of lysozyme it was reported that lithium was the best precipitant, followed by sodium, and then cesium.[14] In this study at 0.5 mol·L^{-1}, all salt types lowered the solubility of mBLA by approximately 20 % at pH 6 and by 10 % to 20 % for cesium and lithium at pH 8 (cf., the salt-free case; Figure 2A). At pH 7 however, the addition of lithium nitrate led to a

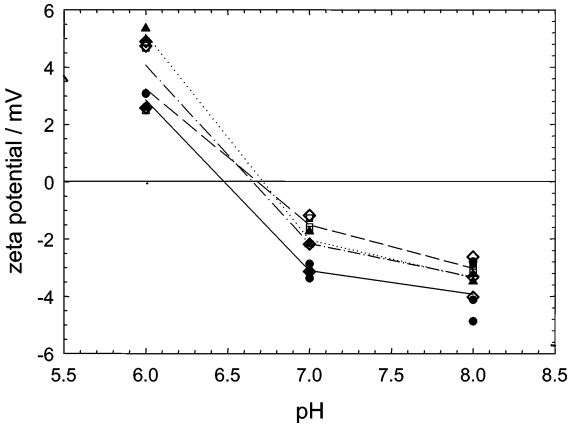

Figure 4. Influence of salts on the ζ potential of mBLA as a function of pH: ●, influence of sodium thiocyanate; □, sodium sulfate; and ▲, lithium nitrate. Salt concentrations were 0.1 mol·L^{-1} and buffered in MES, HEPES, and boric acid, 10 mmol·L^{-1} of each. ◇, For comparison, ζ potential of the salt-free mBLA solution, also buffered in MES, HEPES, and boric acid, 10 mmol·L^{-1} of each.

very slight reduction in solubility, whereas sodium conversely increased the solubility (Figure 3B) to a small extent (cf., the case without added salts). This said, the differences between the three nitrate salts at pH 7 were minimal and within the data spread, and we conclude that the influence of the tested cations on mBLA solubility is small.

(iii) Effects of Salts on the ζ Potential of mBLA. In Figure 4, the ζ potentials measured for mBLA solutions without added salts and with 0.1 mol·L^{-1} sodium sulfate, sodium thiocyanate, and lithium nitrate are shown. In all of the cases examined ζ potential measurements confirmed that mBLA carried a net positive charge at pH 6 and net negative charge at pH 7 and pH 8 (Figure 4). The ζ potential of mBLA was measured using lithium nitrate rather than sodium nitrate since it was found that 0.2 mol·L^{-1} lithium nitrate increased the pI of a structurally related amylase (with approximately 85 % sequence homology and of comparable purity) by approximately 0.7 pH units. We attributed this finding to strong binding of the lithium ion to oppositely charged amino acid residues at the protein surface. In stark contrast, in the present study, substantial changes in mBLA solubility (Figures 2 and 3) and pI (Figure 4) were not observed in the presence of lithium. The effect the different ions induce on the solution properties thus seems to be protein specific and must be inspected carefully for each system being studied. Interestingly, by considering the weighted contributions of each band as determined by gel densitometry, we estimate an average pI of 6.6, which agrees surprisingly well with the pI of 6.7 determined from ζ potential measurements conducted on the salt-free mBLA solution. The ζ potential is based on the average electrophoretic mobility of all protein molecules in solution and leads to one average pH of zero ζ potential, even in the presence of a mixture of proteins of very different structure and pI. Such an average pI is not straightforward to determine by other methods such as isoelectric focusing, particularly when the influences of various salts are to be studied. However, such average pI values are perhaps more relevant for solubility measurements given that solubility is a result of all forces interacting within all the substances present in a particular solution.[14]

At pH 6 the addition of 0.1 mol·L^{-1} sodium thiocyanate resulted in the lowest charge on mBLA followed by sodium sulfate and then lithium nitrate. In other words, lithium nitrate resulted in the most negatively charged protein at pH 6. At pH 7 and pH 8, the lowest net charge on mBLA was observed with sodium sulfate. The presence of thiocyanate yielded the most highly charged protein, followed by lithium nitrate, and then the protein without added salt (Figure 4). Although we have not examined the effects of sodium nitrate, we can anticipate the behavior of this salt, given our earlier demonstration that the cations play a relatively minor role in determining mBLA solubility. Thus, we expect a general shift in the counterions associating with the protein will occur, from the cation sodium at pH 8 and pH 7 to the different anions employed (i.e., thiocyanate, nitrate, and sulfate) at pH 6. It is interesting to note that these trends mirror the above solubility data (for 0.1 mol·L^{-1} salts) exactly (Figure 2 B). In both, the solubility of mBLA at pH 7 and pH 8 in the presence of thiocyanate is much higher than in the presence of nitrate, which in turn is higher than sulfate. At pH 6 there was no significant difference in mBLA solubility for the three tested salts, as is reflected by the ζ potential measurements in Figure 4. The ζ potential measurements also suggested that sodium thiocyanate was capable of changing the pI of mBLA toward lower pH values (i.e., from 6.7 to 6.5). Shifts to lower pH values indicate anion binding whereas the opposite is true for cations.[21] Thiocyanate binding to mBLA can therefore be assumed and is in keeping with reports that the thiocyanate ion binds more tightly and in greater numbers to protein molecules compared to chloride ions, regardless of the sign of the protein net charge.[22] The data suggest that the solubility of mBLA in the presence of low concentrations of salts (i.e., at 0.1 mol·L^{-1}) is controlled by the adsorption of ions to the charged groups of the protein to produce net neutral protein species that crystallize. At higher salt concentrations, the ability of the ions to affect the water structure is the more dominating factor. This is also in accordance with the description given by Curtis et al.[10,11]

The ζ potential is often used to determine the pI of macromolecules or particulate systems, for characterization of the binding behavior of additives, and to estimate the colloidal stability of a system of interest. Solutions that are characterized by ζ potentials > 30 mV (either positive or negative) are generally regarded as stable. Below this value, phase separation processes are likely to occur.[23] The ζ potentials measured for mBLA were < 6 mV, which is much lower than the proposed threshold of 30 mV, indicating an enhanced likelihood of precipitation processes. Indeed, the mBLA system examined is very prone to precipitate formation, either in amorphous or preferentially in crystalline form. Although we have not examined this experimentally, we believe it will be difficult to reach ζ potentials > 30 mV given the limited colloidal stability of mBLA between pH 6 and pH 11. However, because the ζ potential is independent of protein concentration,[16] it cannot indicate conditions in which the solution is supersaturated, which is a necessary requirement for predicting the outcome of crystallization processes. In this context, measurements of ζ potential can only therefore be employed to identify process conditions with increased or reduced risk of phase separation.

In the Introduction, it was discussed that previous workers have proposed that the order of the influence of anions on protein solubility is reversed when crossing the pI to change the net charge on a protein and that this order reversal is a generic phenomenon.[9,13] Although similar tendencies were observed in this study, the data did not show clear evidence of such a general phenomenon with the mBLA preparation and salts used here. Thus in the following discussion the reasons for the behavior observed are examined. The results show that electrostatic interactions are involved in the solubility behavior seen, since the pH giving the lowest solubility was in good

agreement with the pH of zero ζ potential. The data indicates that electrostatic effects dominate at least up to a salt concentration of 0.1 mol·L^{-1} and probably still at 0.5 mol·L^{-1}.[24] Electrostatic interactions of charged groups on the protein molecule with its surroundings vary with the pH of the solution. The results also suggest that the presence of salts can influence both the pI as well as the number of charged groups due to ion binding to appropriate residues on the protein surface and due to changes of the dissociation constants of acidic or alkaline protein groups.

According to Curtis et al.[10,11] the influence of salts on protein solubility depends both on the position of the salts in the Hofmeister series (the lyotropic series) or by the electroselectivity series. In the former, the series is given as sulfate > acetate > chloride > iodide > nitrate > thiocyanate, the corresponding series for cations is magnesium > sodium > potassium > lithium > ammonium > cesium.[5] It lists the influence of the ions on the interaction between protein and the solvent, here the bulk water, which affects solubility. Salts change the chemical potential of the protein in solution by affecting the partition of water between the protein and the salt-ions. Usually, the protein has a higher affinity for water than for the salt, which leads to the formation of a precipitant-depleted zone (or hydration layer) near the protein surface (preferential hydration) that is thermodynamically unfavorable. To decrease the area of the precipitant-depleted zone, the protein molecules associate, which leads to a decrease in solubility.[25] The degree of the preferential hydration is steered by the characteristics of the salt ions involved. Kosmotropic ions like sulfate are more likely to be excluded from the protein surface so that they are more effective in inducing the association of protein molecules than chaotropes.

The electroselectivity series describes the affinity of an ion to bind to an anion-exchange resin, However, it also reflects the ability of the individual ions to modify the charge of the protein surface, either by forming ion pairs with charged amino acid residues or by binding to the exposed peptide groups.[10,11] For monovalent anions, the electroselectivity series is the inverse of the Hofmeister series.[10] According to Collins,[26] the ability of ions to form ion pairs with specific groups on the surface of the proteins depends on the absolute free energy of hydration of both charges and only oppositely charged ions with matching absolute free energies of hydration form strong ion pairs. The absolute free energy of thiocyanate matches closely that of the positively charged amino acid side chains[27] and is furthermore able to bind to peptide groups,[10] whereas sulfate poorly matches any of the positive charges on the protein surface and is not able to bind to the peptide groups.[11,26] The electroselectivity series will prevail at low salt concentration, whereas at higher salt concentrations, the effects of the two series are competing as the increased ionic strength enhances the interaction between the hydrophobic areas on the proteins.[11,27] Following these theories, the results presented above can be explained as follows:

At pH 8, the net charge of mBLA is negative, and here thiocyanate had a solubilizing effect on the enzyme, whereas sulfate decreased the solubility. Furthermore, the ζ potential measurements indicated that the addition of thiocyanate led to an increase in the negative charge of the amylase at a concentration of 0.1 mol·L^{-1}. Hence, one could explain the observed results at this pH by the ability of thiocyanate to form ion pairs with positively charged amino acid side groups or peptide groups, thereby increasing the net negative charge of the protein (as also shown by the ζ potential measurements), which would lead to an increased repulsion between the protein molecules. In contrast, the reduction in mBLA solubility caused by sulfate at pH 8 is most likely best explained by its ability to interact strongly with water. The sulfate is thus preferentially excluded from the surface of the protein, which then will tend to associate to reduce the solvent exposed surface area.

At pH 6, the mBLA net charge is positive, given the weighted average pI of 6.6 measured. Here the ζ potential data showed that binding of the thiocyanate to the protein reduced the net surface charge and thus reduced the electrostatic repulsion between the protein molecules, which caused a (slight) reduction in solubility at the lower salt concentration level relative to the effect of sulfate. Here the salts follow the electroselectivity series. However, at the higher salt concentration level, the electrostatic interactions between the proteins are screened, the protein−protein interaction does not depend on the net charge of the protein but depends more on the ability of the salts to bind water, and the effect of the anions on mBLA solubility follows the Hofmeister series.

It is commonly accepted and confirmed by this study that cations play a minor role on the solubility of proteins compared to anions.[9] This phenomenon aggravates the identification of a reversal of the Hofmeister series for cations, which should be expected if ion pairing and charge screening are dominant over effects caused by preferential hydration (electroselectivity). In the case of the cations, none of the series (i.e., the lyotrophic or electroselectivity series) could be confirmed. In addition to electroselectivity or ion pairing effects, the picture is further complicated by the ability of lithium to form strong complexes with amides.[26,28]

Conclusions

The current study demonstrated that concepts developed on highly purified proteins are only partly applicable to systems of minor purity consisting of various isoforms such as mBLA. In particular, no clear evidence for the reversal of the Hofmeister series for anions and cations upon changing polarity of the protein net charge was found. Deviations from the solubility behavior predicted for highly purified proteins (e.g., upon salt addition) should thus be expected. It is, however, necessary to study more proteins to determine if the tendencies reported here are general. Solubility measurements are tedious but inevitable to correctly characterize the solution properties of the system of interest, which is crucial for the development of reliable crystallization processes for bulk enzyme recovery. Nevertheless, we found that the ζ potential was helpful for describing the solution properties of mBLA, particularly in the presence of impurities and isoforms. This method may thus be useful to speed up cataloguing of the effects of salts and pH on different proteins. Measurements of the ζ potential enabled the rapid determination of the protein's pI and could, although limited to low ionic strengths, in qualitative terms correctly predict the impact of the tested salts on solubility. Thus, critical conditions (e.g., of low solubility) and formulation additives that enhance the colloidal stability of a given protein could potentially be rapidly identified. However, ζ potential measurements cannot replace solubility experiments, but rather could potentially be used to reduce the number of them by focusing on critical conditions and thus accelerate the development of bulk enzyme recovery processes.

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