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Lysozyme purification from tobacco extract by polyelectrolyte precipitation

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Abstract

Tobacco is widely used as a model plant for feasibility studies of recombinant protein production from transgenic plants. However, dealing with large quantities of biomass to recover recombinant proteins is a challenge for down-stream processing. In this study, the effect of isoelectric precipitation on native tobacco protein was first studied. Among the three acids studied, hydrochloric acid is shown to be more effective than acetic or citric acid, and at pH 4, 60% of native tobacco protein was precipitated by HCl. Egg white lysozyme was used as the model protein to test the feasibility of polyelectrolyte precipitation in protein recovery from tobacco extract. Precipitation of lysozyme at pH 7 was shown ineffective probably because of the interference of polyphenolic acids. However, after isoelectric precipitation at pH 5 poly(acrylic) acid (PAA) was shown to precipitate 85% of the soluble lysozyme when the polymer dosage was increased to 1.5 mg polymer/mg lysozyme, while negligible amounts of native tobacco protein was co-precipitated. Lysozyme precipitation by PAA in tobacco extract obtained at pH 5 was also studied, and lysozyme yield was significant improved.

Keywords: Precipitation; Isoelectric; Polyelectrolyte; Lysozyme; Transgenic tobacco; Recombinant protein

1. Introduction

Plant systems are very cost-efficient in biomass production, and they do not carry viruses and other pathogens dangerous to humans such as human immunodeficiency virus (HIV), prions, hepatitis viruses and so on. In addition, the protein synthesis pathway is conserved between plants and animals, so plants can fold and assemble recombinant human and other mammal proteins efficiently to produce bioactive proteins [1,2]. Thus, transgenic plants have been widely studied as alternative sources to other expression systems, such as microbial, animal cell, or transgenic animal expression systems, to produce recombinant proteins.

Tobacco was the first flowering plant to be transformed and regenerated [3], and it is one of the easiest plants to be genetically engineered. Because of its ease of culture and transformation, it is still used as an alternative model system for studying plant development. Tobacco has also been recognized as an ideal production system for plant based biologics [4,5]. After the appearance of the unapproved Starlink *Bacillus thuringiensis* (Bt) toxin from recombinant corn in the food supply, it is increasingly obvious that as a non-food and non-feed crop tobacco is a much safer alternative for production of pharmaceutical proteins. Furthermore, protein production from transgenic tobacco can be easily scaled-up. Tobacco's biomass (leaf) yield can reach as much as 170,000 kg/ha, and each plant can produce about 150,000 seeds for reproduction and scale-up [5]. Tobacco has also been shown to successfully express numerous therapeutic proteins and many monoclonal antibodies. However, commercialization of tobacco as a host for recombinant protein production is dependent on the development of cost effective and efficient methods to handle huge amounts of biomass. Since the overall cost of a protein's purification is mainly determined by the efficiency of the initial recovery and purification, extraction and initial purification procedures

Abbreviations: BCA, bicinchoninic acid; BSA, bovine serum albumin; CMC, carboxymethyl cellulose; DHB, dihydroxybenzenes; PAA, poly(acrylic) acid

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of recombinant proteins from tobacco need to be developed.

Because it is a simple unit operation, relatively inexpensive and straightforward to be scaled-up, precipitation is often used in the early stages of protein purification processes for protein recovery and purification. Both isoelectric and polyelectrolyte precipitation have been shown to be effective and surprisingly selective in separating proteins from the extracts of various plant hosts [6,7]. Isoelectric precipitation is achieved by adjusting the pH of a protein solution and is based on that a protein's solubility is at minimum at its p*I*, and proteins form aggregates during isoelectric precipitation mainly through hydrophobic interaction. However, it may result in loss of activity due to denaturation if the target protein is precipitated. On the other hand, protein precipitation by polyelectrolyte, which is based on the electrostatic interaction between a charged polymer and oppositely charged proteins, has additional advantage because the precipitated proteins maintain their bioactivities and can be readily resuspended in aqueous solutions, often at higher concentrations [8].

Zaman et al. used isoelectric precipitation to remove native canola protein from lysozyme in canola extract [6]. They reported that at pH 5, 70% of native canola proteins precipitated, while 90% of lysozyme remained in solution. In the same report, however, precipitation of lysozyme with polyelectrolytes sodium hexametaphosphate (Glass H) and poly(acrylic acid) (PAA) at near pH 7 was unsuccessful probably due to interference of canola components in the extract. PAA was also used to separate lysozyme from egg-white but with much greater success [9]. Nine-fold higher specific activity was achieved, which also indicates that little denaturation takes place during precipitation. It was also reported that lysozyme precipitation is pH dependent. Moreover, carboxymethyl cellulose (CMC), another negatively charged polyelectrolyte at neutral pH, was studied to recover protein from whey [10], although the goal was to recover and concentrate the total protein rather than a target protein.

In this study, the precipitation profile of native tobacco protein under different pH was first determined. Egg-white lysozyme, molecular mass 14,000 and p*I* ca. 10.5, was then used as a model protein to test the effectiveness of polyelectrolyte precipitation for selective lysozyme recovery from tobacco extract. Three polyelectrolytes, PAA, CMC, and Glass H, were studied, and the influences of pH and extraction condition on precipitation were also reported.

2. Materials and methods

2.1. Materials

Unless specified, all chemicals including egg-white lysozyme were purchased from Sigma (St. Louis, MO, USA). Bovine serum albumin (BSA) and bicinchoninic acid (BCA) assay reagents were purchased from Pierce (Rockford, IL, USA).

PAA was purchased from Polysciences Inc. (Warrington, PA, USA). Its molecular mass is ca. 500,000. CMC was obtained from Sigma, and its molecular mass is ca. 700,000 with degree of substitution (DS) of 0.9. Glass H, sodium hexametaphosphate with an average of molecular mass of 2200, was donated by Astaris (Lawrence, KS, USA).

Burley tobacco was grown at the Virginia Tech Southern Piedmont Agriculture Research and Extension Center, Blackstone, VA during the summer of 2001. Plant tissue was stored on ice during transport to Blacksburg then stored at −80 °C.

2.2. Tobacco extract

Tobacco extract was obtained following reported protocol [11]. Frozen burley tobacco leaves were thawed at room temperature until soft. Deionized water was used to rinse soil and other contaminants from the leaves. The leaves were then blotted dry with paper towels. The total mass of the leaves was measured. The stalk was separated from the leaf tissue and discarded. The remaining leaf tissue was weighed and blended. The tissue was then separated into approximately 3 g samples. Buffer was added to the samples in the ratio of 10 mL buffer:1 g leaf. For isoelectric precipitation, 50 mM, pH 9 Tris buffer was used. For polyelectrolyte precipitation, 50 mM pH 7 sodium phosphate (NaPi) was used. The samples were then homogenized and weighed.

The homogenized samples were allowed to stand at room temperature for 15 min. Then, the samples were centrifuged at 4 °C for 20 min. After centrifugation, the supernatant was collected and then filtered.

2.3. Isoelectric precipitation

Filtered tobacco extract was separated into 2 mL samples, and the pH of the extract was measured. Each sample was titrated with acid until it reached desired pH. Precipitation was tested at pH 3–8. Acids used were 0.2 M HCl, 0.5 M citric acid, and 0.5 M acetic acid. Triplicates were performed for each acid at each pH. Samples were then centrifuged at 4 °C and 12857 × *g* for 20 min.

After centrifugation, the protein content of the supernatant of each sample was measured using BCA assay. Prior to the assay, samples with pH 5–9 were diluted (2× and 10×) because protein content was higher than the linear range of the assay. Samples were diluted in 50 mM buffer with the same pH to prevent additional protein precipitation. For pH 5, citrate-phosphate buffer was used. Buffers from pH 6 to 8 were prepared using sodium phosphate, and the pH 9 buffer was prepared using Tris base.

2.4. Polyelectrolyte precipitation with tobacco extract obtained at pH 7

Tobacco extract was obtained with pH 7 NaPi buffer. Total tobacco protein concentration is ca. 3.5 mg/mL. Lysozyme was spiked into tobacco samples with a final concentration

of 0.2 mg/mL. The total volume of each sample was 1.4 mL. For PAA and Glass H, the polymer stock solutions were 14 mg/mL in 50 mM NaPi, pH 7, and the amounts of polymer used were 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.5 mg/mg lysozyme. The 50 mM NaPi buffer at pH 7 was used to make final total volumes equal. For CMC, the stock solution was 4.7 mg/mL, and the amounts of polymer used were 0, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/mg lysozyme.

After addition of polyelectrolyte, samples were vortexed and allowed to precipitate for 15 min and then centrifuged in a Marathon centrifuge for 15 min at 10,000 rpm. Supernatant was separated from precipitant by pipetting. Protein concentration was measured with BCA assay. Concentration of lysozyme was measured by SDS-PAGE gel analysis. A lysozyme activity assay was attempted to measure lysozyme content, but was unsuccessful. All experiments were carried out at room temperature.

Protein precipitation with PAA was also attempted at pH 5. For this study, lysozyme-spiked tobacco extract at pH 7 was first titrated to pH 5 with HCl. After isoelectric precipitation, polyelectrolyte precipitation with PAA was performed with concentrations of 0, 0.3, 0.6, 0.9, 1.2, and 1.5 mg PAA/mg lysozyme originally added. Again, protein concentrations were analyzed using BCA assay and SDS-PAGE analysis.

2.5. Polyelectrolyte precipitation with tobacco extract obtained at pH 5

Tobacco protein extract was obtained with 50 mM citrate–phosphate buffer, pH 5. PAA stock solution was 14 mg/mL in the extraction buffer, and the dosage of PAA studied was same as above. Overall lysozyme concentration in the spiked extract was 0.2 mg/mL, and the spiked extract was allowed to stand at room temperature for 15 min then vortexed and filtered before PAA precipitation. The precipitation protocol is same as above.

2.6. BCA assay

Protein concentration was determined by bicinchoninic acid assay [12]. Bovine serum albumin was used as standard. Fifty microliters of sample was mixed with 1 mL of the working reagent and incubated at 37 °C for 35 min. The samples were allowed to cool to room temperature before absorbance was measured at 562 nm.

2.7. SDS-PAGE electrophoresis

Samples were prepared with 2× sample buffer, and the final concentration of β-mercaptoethanol in the samples was 1%. The sample/buffer mixture was incubated at 85 °C for 2 min. The gels were run for 1.5 h at 125 V.

After running, the gels were washed and stained with Coomassie-blue. The gels were then scanned with a BioRad densitometer, and analyzed using Quantity One Software.

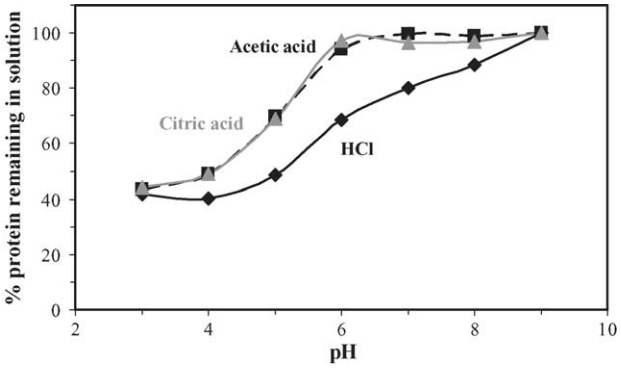

Fig. 1. Isoelectric precipitation profiles of native tobacco protein. The *y*-axis indicates the weight percentage of tobacco protein remaining in the solution after precipitation at different pH. The extract pH was titrated with different acids.

3. Results and discussion

3.1. Isoelectric precipitation

The solubility of tobacco protein was considered as a fraction of the tobacco extract obtained at pH 9, which was assigned to be 100%. It should be noted that the pH of the extract dropped to approximately pH 8.7 after extraction. All results were measured using BCA assay and confirmed with SDS-PAGE gel analysis.

As shown in Fig. 1, different acid has different tendencies in precipitating proteins. The tobacco protein solubility profile obtained with HCl is noticeably different from that obtained with citric acid or acetic acid. For HCl, tobacco protein solubility reached a minimum at pH 4 at ca. 40.2%. For citric and acetic acid, the highest protein separation was observed at pH 3. The minimum solubility was 44.3% for citric acid and 43.3% for acetic acid. Interestingly, the solubility curves seem to converge at pH 3.

For acetic acid, titration to pH 3 from pH 4 required a 10-fold increase in acid added by volume. For citric acid, it required a 4-fold increase. However, this additional acid only improved precipitation about 5%, from 49.0% to 43.3% with acetic acid and from 48.9% to 44.3% with citric acid. The large volume of acetic and citric acid needed to decrease the solution pH is not surprising because the pK_a of acetic acid is 4.74 and the acid is weakly ionized in aqueous solution. Meanwhile, the pK values of the three carboxyl groups in citric acid are 3.13, 4.76, and 6.40, respectively. Moreover, the protein solubility showed a large drop between pH 5 and 6 for all three acids. Insignificant protein precipitation was observed at pH 6 for citric acid and acetic acid, but the protein solubility dropped to about 69% at pH 5. For HCl, the solubility dropped from 68.4% to 48.7% between pH 5 and 6. Further titration to lower pH was not efficient, as the protein solubility at pH 5 was only about 8% higher than the minimum solubility achieved. This result indicates that titration at different pH may be useful in separating proteins that are sensitive to very acidic conditions.

The different tendencies for protein precipitation with different acid may be attributed to the varying ability of the anions (Cl^-, CH_3COO^-, and $CHO(COO)_3^-$) interacting with the positively charged amino acid residues on protein surface. For HCl precipitation, it may be easier for the small chloride ion to interact with the positively charged groups on protein surface to reduce the repulsive electrostatic forces during protein aggregation. From pH 9 to 6, the reduced repulsive effect is significant to promote protein aggregation during isoelectric precipitation by HCl. From pH 6 to 5, the change of solution pH is more significant in inducing protein aggregation for all three acids. At lower pH, the reduced repulsive effect becomes less significant and eventually leads to the convergence of the solubility profiles. In addition, the tobacco protein solubility profile with HCl is very similar to that of canola protein, except that the minimum is occurred at pH 5 for canola protein instead of pH 4 for tobacco protein [6]. For acetic and citric acids, protein precipitation is largely dependent on the pH change. The reduced repulsive effect by the interaction between anions and positively charged groups on protein surface is minimal at elevated pH or absent at lower pH.

3.2. Polyelectrolyte precipitation at pH 7

Tobacco protein extract was obtained with 50 mM NaPi at pH 7 with total concentration ca. 3.5 mg/mL. For all three polymers studied, polyelectrolyte precipitation was unsuccessful. Lysozyme content was measured with SDS-PAGE gel analysis, because lysozyme activity assay proved unreliable. As shown in Fig. 2 (data for CMC precipitation is not shown), the solubility of lysozyme is approximately 30% regardless of the amount or the variety of polymer added to the extract. The variation of lysozyme solubility is likely due to the error in protein quantification by Quantity One.

The low solubility of lysozyme in tobacco extract is unexpected. Zaman et al. spiked 0.2 mg/mL of T4 lysozyme in 1.2 mg/mL of canola protein (lysozyme accounted for 14% of total protein), and almost all T4 lysozyme was soluble at pH 7 [6]. In this study, lysozyme only accounted for ca. 6% of the total protein in spiked samples. However, about 70% of lysozyme was lost due to precipitation. Interestingly, the remaining lysozyme does not appear to interact with either of the polyelectrolytes, PAA (Fig. 2a) or Glass H (Fig. 2b), but both PAA and Glass H have been shown to induce pure lysozyme precipitation [6]. One plausible explanation for this phenomenon is that some components in tobacco extract interact with the positively charged lysozyme to prevent the protein from interacting with the polymers.

The presence of phenolic substances in tobacco may be responsible for the precipitation of lysozyme and shielding off lysozyme from interacting with the polyelectrolytes. Rawel et al. demonstrated that several phenolic substances found in plants, *o*-, *p*-dihydroxybenzenes (DHB), and gallic acid form complex structures with lysozyme [13]. The isoelectric range of unmodified lysozyme is between p*I* 10.5 and 11.3. In the presence of these substances, the isoelectric range of lysozyme shifts to pH range 6–7 (*o*-DHB), pH range 3.5–9 (*p*-DHB), or pH range 3–9 (gallic acid). This means that in the presence of any of these substances or other commonly found polyphenolic acids in tobacco leaves, such as chlorogenic acid [14], lysozyme is mostly insoluble or uncharged (or negatively charged) at pH 7. Thus, precipitation of lysozyme in tobacco extract at pH 7 with negatively charged polyelectrolytes becomes impossible.

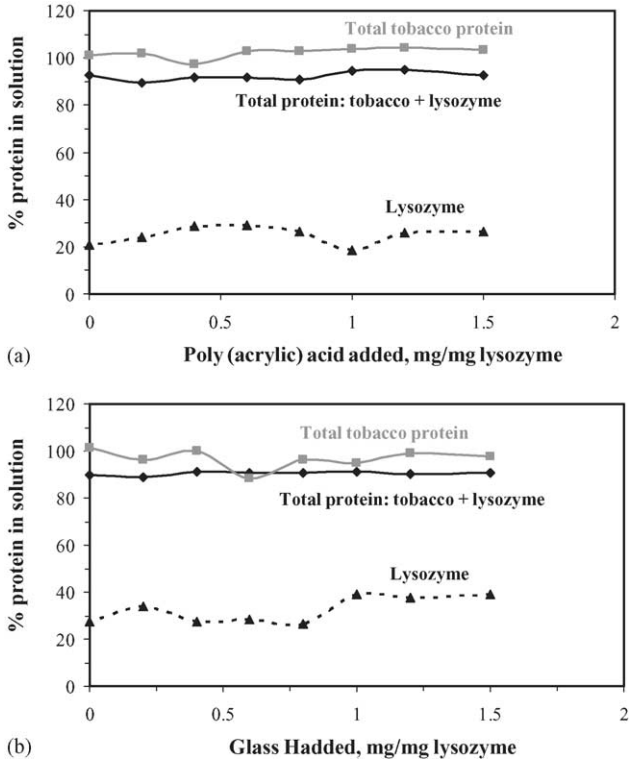

Fig. 2. Protein solubility profile after addition of (a) PAA and (b) Glass H. Lysozyme concentration was obtained by Quantity One volume quantity analysis of SDS-PAGE gels. Total tobacco protein and total protein concentrations were obtained by BCA assay. The *y*-axis indicates the weight percent of corresponding protein remaining in aqueous solution. The total tobacco protein solubility curve was obtained with non-spiked tobacco extract.

On the other hand, total tobacco protein (unspiked) and the total protein including lysozyme do not vary with the addition of polyelectrolytes. The fact that no significant native tobacco protein was precipitated with either of the polyelectrolytes shows that most of the tobacco proteins are of acidic nature. Our previous work showed that the overall tobacco cellular environment is acidic, and more acidic proteins are present in aqueous tobacco extract [11]. This actually presents opportunities for selective recovery of basic proteins, such as lysozyme, and it needs to be taken into consideration when tobacco is considered for recombinant protein expression. However, the interference from the presence of polyphenolic compounds needs to be overcome.

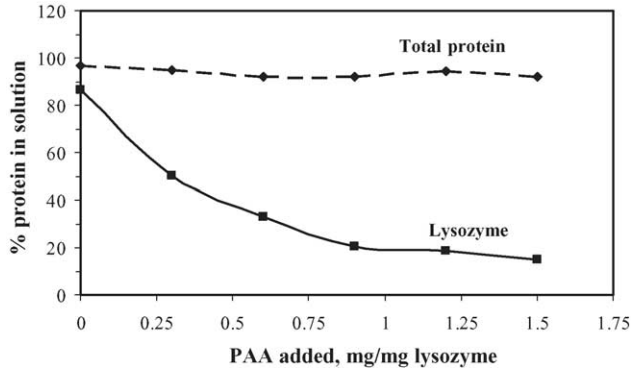

Fig. 3. Lysozyme precipitation in spiked tobacco extract at pH 5 by PAA. Tobacco extract was obtained at pH 7. The *y*-axis indicates the weight percentage of protein remaining in the solution after precipitation.

3.3. Polyelectrolyte precipitation at pH 5 with tobacco extract obtained at pH 7

To circumvent the problems posed by the presence of phenolic substances in tobacco extract, lysozyme-spiked tobacco extract was first titrated to pH 5 and then PAA was added. As shown in Fig. 3, after first titrating spiked tobacco extract to pH 5, PAA becomes effective in precipitating lysozyme. It should be noted that only ca. 33% of the originally spiked lysozyme remained in the supernatant after pH adjustment. Nevertheless, about 85% of the lysozyme remaining in solution at pH 5 was precipitated by addition of PAA (1.5 mg PAA/mg total lysozyme), and the amount of lysozyme precipitated increases with the amount of PAA added. Meanwhile, over 92% of tobacco protein after pH adjustment remained in the supernatant. The overall lysozyme recovered by PAA precipitation (Fig. 4), however, is only ca. 29% due to the loss of lysozyme at pH 7. Based on the results in the previous section, it is safe to assume that the loss of lysozyme occurred before the titration even started.

Compared to the precipitation carried out at pH 7 where none of the three polyelectrolytes worked, however, this still is a noticeable improvement. The enrichment ratio of lysozyme precipitated at pH 5 is ca. 11. The reason for this significant improvement could be that the interactions between lysozyme and polyphenolic compounds are weakened or disrupted at pH 5 to allow PAA–lysozyme interaction to take place. Precipitation studies with Glass H and CMC were not performed.

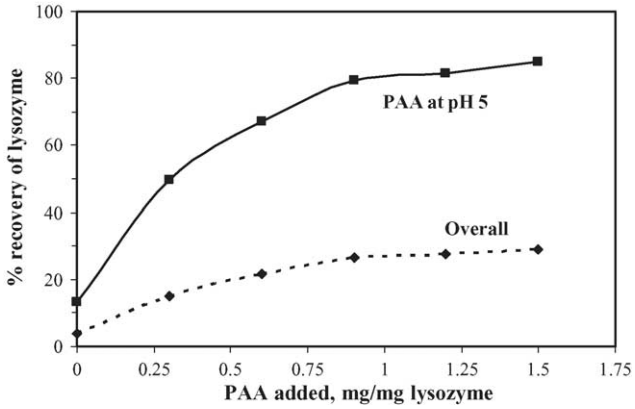

Fig. 4. Recovery of lysozyme at pH 5 by PAA precipitation and overall recovery in reference to total lysozyme spiked at pH 7. Tobacco extract was obtained at pH 7.

3.4. PAA precipitation at pH 5 with tobacco extract obtained at pH 5

Since the above results showed that at pH 5 lysozyme–PAA interaction was enabled but with low recovery due to the protein loss as pH 7, studies were carried out to test if the extraction condition would affect the lysozyme recovery. Total tobacco protein extracted at pH 5 is ca. 2.5 mg/mL, which is ca. 30% less than that of pH 7 extract. This 30% total tobacco protein decrease agrees with the isoelectric titration curve shown in Fig. 1. Fig. 5 shows the total protein and lysozyme remained in the supernatant during PAA precipitation and the overall recovery of lysozyme in the precipitate in reference to total lysozyme spiked. It is clear that most of the spiked lysozyme (ca. 75%) remained in pH 5 tobacco extract. Since lysozyme is a basic protein, extracting total tobacco protein at lower pH will not likely affect the amount of lysozyme extracted (for recombinant protein extraction). Thus, this increased lysozyme solubility proves that by selecting extraction conditions at low pH, the possible basic protein precipitation caused by co-extracted polyphenolic compounds can be significantly reduced.

The lysozyme and total protein solubility profiles are similar to those shown in Fig. 3. The overall yield of lysozyme from PAA precipitation is significantly improved. At the dosage of 1.5 mg PAA/mg of lysozyme, more than 53% of the originally spiked lysozyme was recovered in the precipitate in comparison with 29% in Fig. 4. Comparing Figs. 3 and 5,

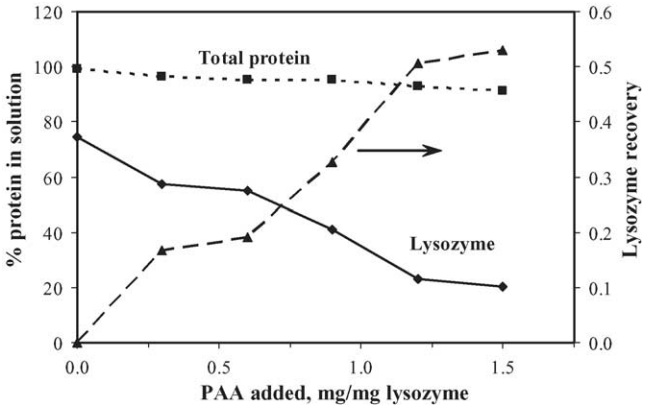

Fig. 5. Precipitation of lysozyme by PAA at pH 5 with tobacco extract obtained at the same pH. The arrow indicates that the line is referred to the *y*-axis at the right for overall lysozyme recovery. The other two lines are referred to the *y*-axis at the left.

it is clear that more PAA is needed to precipitate lysozyme in pH 5 extract simply because there is more lysozyme present. If the dosage of PAA was increased, it is expected that more lysozyme would be precipitated and higher lysozyme yield would be obtained. The calculated enrichment ratio is ca. 8, which is lower than that obtained in the previous section because of the significantly higher lysozyme concentration in the tobacco extract. In summary, by extracting tobacco protein at pH 5, lysozyme becomes more soluble in the extract, and PAA is efficient in precipitating lysozyme for selective recovery and purification.

4. Conclusion

Different acids tend to precipitate tobacco protein differently during isoelectric precipitation, and HCl is most efficient. The difference may be attributed to the ability of the chloride ions to interact with the positively charged groups on protein surface to reduce the electrostatic repulsion during protein aggregation. However, this effect diminishes at lower pH. The maximum precipitation with any of the acids was observed at pH 4 with HCl, where ca. 60% of tobacco protein was precipitated.

Egg-white lysozyme was used as a model protein to test the utility of polyelectrolyte precipitation from tobacco extract. At pH 7, most of lysozyme was precipitated after spiking, and neither of the polyelectrolytes was effective at this pH. The plausible explanation is that the polyphenolic compounds present in the extract interact with lysozyme to form more hydrophobic and more acidic complexes, and these complexes cause lysozyme to precipitate and prevent it from interacting with the polyelectrolytes. After titrating the spiked tobacco extract to pH 5, PAA was shown to be efficient in lysozyme precipitation with high lysozyme enrichment. Further improvement was achieved with tobacco protein extract obtained at pH 5. More than 75% of the spiked lysozyme remained in the solution, and the overall lysozyme yield by PAA precipitation was improved from 29% to more than 53%. This shows that with properly selected conditions, basic proteins, especially recombinant proteins expressed in transgenic tobacco, can be extracted and selectively separated by polyelectrolyte precipitation. This should provide guidance for process design for recombinant protein recovery and purification from transgenic tobacco.

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