ISOLATION AND PURIFICATION OF JACALIN FROM ARTOCARPUS HETEROPHYLLUS LAM

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Abstract: This paper presents investigation results of saturation conditions needed for purification of jacalin lectin from the extract seeds of *Artocarpus heterophyllus* by ammonium precipitation and affinity chromatography on Galactose-Affi gel Hz. Three different aspects of parameters encompassing the percentage of saturation of ammonium sulfate precipitation, the presence of ammonium sulfate on Lowry method and the suitable galactose concentration for optimum elution of the protein from Galactose-Affi gel Hz were investigated. With three different sets of fractional saturation of jacalin purification using ammonium sulfate precipitation, the maximum yield of 0.463 g/g was achieved at 0-90% saturation range in the absence of dialysis. Maximum yield of 0.425 g/g was obtained at 30-60% and 0-90% saturation range in the presence of dialysis. The result from this work also indicates that excessive quantity of NH_4SO_4 interferes with Lowry method for protein determination substantially. The 0-90% saturation range was found to be more potentially appropriate for large scale application than 30-60% saturation, since the former involves only 1 step NH_4SO_4 addition. From the affinity chromatography, elution of 0.2 M galactose (in 0.15 M NaCl) from Galactose-Affi gel Hz produced the maximum peak profile and jacalin concentration. A reduction or increase in galactose concentration of more than 0.2 M did not increase concentration of purified jacalin purified using this method.

Keywords: *Jacalin-lectin purification, Lowry method, ammonium precipitation, protein determination.*

1. INTRODUCTION

Jackfruit known scientifically as *Artocarpus Heterophyllus Lam* is a tropical plant that can be found in abundance in warm climate countries such as India and in most ASEAN countries such as Malaysia. Jackfruit is a useful plant from which humans can benefit from regardless of whether it is for food, medicine or farming purposes. One Malaysian company called Inter-Crystal Agritech, has been working very closely with USM to produce new variety of jackfruit. One of the most successful ranges of products is the "nangka madu" collection that consists of CJ1, CJ2, CJ3, CJ4, CJ5 and CJ6.

Although the uses of jackfruit flesh have been known well, the seeds are now being studied too. Besides being rich in carbohydrates, the seeds have been found to contain lectin (carbohydrate-binding protein). Several researchers have mentioned the presence of numerous lectin found in the seeds such as jacalin [1,2], KM+ [3,4] and frutalin [5], each showing their own specificity for a

particular sugar. Recent advancement in medicine has shown that jacalin inhibits HIV-1-infection of lymphoid cells *in vitro* without preventing virus binding on the host cell [6]. Jacalin is also a useful tool for studying serum and secretory immunoglobulin A (IgA) and can be used for the isolation of human immunoglobulin A1 by affinity chromatography [7]. Jacalin is also used for the purification of glycoproteins containing O-linked oligosaccharides [8] and as histochemical detection of the Thomsen-Friedenreich antigen tumours [9]. In the market, jacalin is sold in salt free lyophilized powder for about Euro 45 (RM 158) for 10 mg. Due to its good price and vast benefits, it is commercially attractive to purify jacalin from jackfruit seed as jackfruit is abundant in Malaysia the whole year through, the seeds can be obtained easily and jacalin can then be purified using various methods involving such steps as solubilization, stabilization, isolation and concentration. Chromatography methods that have been used to purify jacalin were anion exchange high performance liquid chromatography (HPLC) [10] and affinity chromatography. Affinity chromatography has been the preference due to the specificity of jacalin towards galactose, which is used as the ligand on the affinity matrix. Focus of this work has been to purify and extract jacalin from CJ1 jackfruit, a local variation obtained from Inter-Crystal Agritech farm, by means of affinity chromatography. The optimum operating conditions for affinity chromatography method were also studied. Instead of applying conventional methods for jacalin extraction [1, 7, 11, 12] that employed phosphate-buffered saline (PBS) and IgA-Sepharose 4B column, this work employed sodium chloride solution as a substitute for PBS and Galactose-Affi gel Hz for IgA-Sepharose 4B column.

2. EXPERIMENTAL

2.1 Raw material

Jackfruit of variety CJ1 was obtained from Inter Crystal Agritech. The seeds were separated from the flesh and frozen at –20°C until use.

2.2 Chemicals

The chemicals used were analytical grade. Sodium Chloride (NaCl) and Sodium Carbonate (Na_2CO_3) were purchased from R&M Chemicals. Copper (II) Sulfate Pentahydrate ($CuSO_4.5H_2O$), Sodium Hydroxide (NaOH), Folin reagent and Sodium Acetate ($C_2H_3NaO_2$) were from Merck. Ammonium Sulfate Salt (NH_4SO_4) and Sodium Periodate ($NaIO_4$), were purchased from BDH Chemicals. Other chemicals such as Sodium potassium tartrate were obtained from AJAX Chemicals, Bovine Serum Albumin from AMD, Galactose ($C_6H_{12}O_6$) from Sigma Chemical Co., Affi-gel Hz gel – crosslinked agarose from Biorad and Glycerol anhydrous ($C_3H_8O_3$) from J.T.Baker.

2.3 Lectin extraction

In the preparation of jackfruit flour, frozen seeds were thawed, washed, de-hulled and cut into small pieces before being dried under the sun for 12 hours. The dried seeds were ground into powder using an electric blender and further dried for 2 hours before being stored in bottles for further use. Three sets of ammonium sulfate precipitation using different percentage of saturation were prepared. 25 g of seed flour was stirred with 100 ml of 0.15 M NaCl in a beaker using a magnetic stirrer. The suspension was left at room temperature for 30min. Later, it was filtered with Whatman filter paper into a beaker (541-hardened ashless, 90 cm diameter). The filtrate was centrifuged for 20 min at 10000×*g*, 7°C. The supernatant from each tube was carefully decanted into a 250ml-graduated cylinder. The volume of crude extract was recorded and the pellet at the

bottom of the centrifuge tube discarded. The supernatant was transferred to a 600 ml beaker. The amount of NH_4SO_4 salt to be added to obtain the desired percentage of saturation was calculated according to precipitation table [13]. NH_4SO_4 salt was added to the supernatant with continuous stirring throughout the salt addition until complete equilibrium between dissolved and aggregated proteins was achieved. The solution was centrifuged for 20min at 10000×g, 7°C. The supernatant was poured into a 250 ml graduated cylinder and the volume recorded.

2.4 Dialysis

A cellulose dialysis tube (Molecular weight cut off: 6000-8000 from Spectra) of about 80 cm was soaked in distilled water for about 2 hours. The total volume of extract obtained earlier was poured into the tube and two knots were tied to seal off the tube. The tube was dialyzed in a beaker against 0.15 M NaCl for 24 hours at 4°C. The dialyzed content was emptied into a graduated cylinder and the final volume noted before it was kept refrigerated at 4^0C for further use.

2.5 Affinity chromatograph column preparation

2.5.1 Coupling buffer preparation

Affi-Gel coupling buffer was prepared by mixing 100 mM sodium acetate and 150mM sodium chloride. The pH for the buffer was corrected to pH 5.5 using acetic acid/sodium hydroxide.

2.5.2 Oxidation of galactose

Galactose was oxidized using the manufacturer's method [14] with some modification. 2 ml stock solution was added to 20 ml galactose and gently mixed for 1 hour. Immediately after oxidation, glycerol was added at a final concentration of 20 mM and was again mixed for 10 minutes. Oxidized galactose was then dialyzed against Affi-Gel coupling buffer at pH 5.5.

2.5.3 Washing the Affi-Gel Hz Gel

With reference to the manufacturer's method [14], Affi-Gel was washed before coupling. In order to remove isopropanol, Affi-Gel was rewashed for several times using coupling buffer.

2.5.4 Coupling of Oxidized Galactose

Coupling was done according to the manufacturer's method [14] for 24 hours at room temperature with adequate mixing. After coupling, galactose-Affi-gel was poured into column. The eluent was collected and volume measured. The gel was then washed with one volume of suitable buffer containing 0.5 M NaCl. As buffer other than the example was used, the column was equilibrated with 10 volumes of 0.15 M NaCl buffer. The column eluent was collected and saved for galactose coupling determination.

2.6 Lectin purification

Purification of lectin was done using the method applied by [5] with some modifications. The column was packed with the galactose- Affi gel Hz gel and equilibrated with 0.15 M NaCl in 10 volumes of this buffer. Extracts of jackfruit flour was flowed through the column at about 5ml/min. Then, the column was washed with 0.15 M NaCl. The column was eluted with 0.2 M galactose in the equilibrium solution. Fractions of eluent from the column was collected. All

fractions were screened using a UV spectrophotometer for absorbance at 280 nm. Elution of the column was continued until A_{280} fell below 0.01.

2.7 Protein Quantification

This was determined by the Lowry protein determination method as described in reference [15], using bovine serum albumin (BSA). As the assay is not linear at high concentrations, concentration of standard and sample should be diluted with distilled water to ensure that they are analyzed on the linear portion of the calibration curve. Readings at 280nm were used to determine the approximate protein content of column eluteas.

3. RESULTS AND DISCUSSIONS

3.1 Ammonium sulfate precipitation

3.1.1 Without dialysis

Ammonium sulfate (NH_4SO_4) is highly soluble in water and actually reduces the water concentration available for interaction with jacalin. Hence, jacalin is precipitated out. The amount of NH_4SO_4 saturation needed was calculated in accordance to [13].

Referring to Table 1, it can be seen that in set 1, for the range of 0–30% saturation there is only a slight yield of jacalin that was purified. Nevertheless, for the range of 30-60% saturation, the amount increases about 3 folds of that for 0–30% saturation. In the range of 60-90% saturation, a drop in jacalin yield was observed. This trend shows that 30% saturation of NH_4SO_4 is not sufficient to precipitate all the jacalin in the extract. A large portion of jacalin, could only be precipitated at the 30-60% saturation range. The large amount of NH_4SO_4 salt added increases the amount of NH_4SO_4 molecule available for contact with the water molecules in the extract. Hence, more jacalin is dehydrated or in other words precipitated. Further addition of NH_4SO_4 from 60% until the 90% saturation could only precipitate the trace amount of jacalin left in the extract.

For the set 2, high yield is obtained initially in the 0-60% saturation range and then only a slight amount of yield is seen for 60-90% saturation.

For the set 3, high jacalin yield is obtained immediately and is somewhat similar to that of the set 2. This is because the amount of NH_4SO_4 added was sufficient to precipitate the majority of jacalin in the crude extract.

For all sets, it can be seen that the total protein of each fraction does not sum up to the total protein in the crude extract. The loss was due to the protein denaturation caused by excessive vigorous mixing. The shearing effect had destroyed the protein conformation.

In general, set 3 in the range of 0-90% saturation of NH_4SO_4, yield the highest fraction purified of about 48% protein, followed by set 1 in the range of 30-60% saturation of NH_4SO_4 and set 2 in the range of 0-60% saturation of NH_4SO_4.

3.1.2 With dialysis

In Table 2, set 1 for ammonium sulfate precipitation with dialysis shows a marginal yield for 0-30% saturation and the yield increases significantly by 3 folds at the range of 30-60% saturation

but decreases to a smaller yield. Set 2 shows a higher yield of purified jacalin for 0-60% range saturation compared to 60-90% range. Set 3 shows, saturation range of 0-90% also showed a high yield just like in the set 3 but at lower magnitude.

In brief, there are similarities for ammonium sulfate precipitation with and without dialysis. First, 30-60% saturation in set 1 gives the highest yield. Second, both of the methods produced lower yield than that obtained in the literature [5]. Different rates of mixing and addition of ammonium sulfate salt were identified to be the primary cause of these differences. Increase of mixing speed increases the rate of dissipation and hence dilution of protein (in this case jacalin) and salt concentrations [16]. Vigorous stirring can cause frothing, in which, proteins that are caught in bubbles are denaturated during the process [17]. Agitation rate also has the potential to cause shear inactivation or partial denaturation of protein [16].

Table 1: Ammonium Sulfate precipitation without dialysis

Trial 1

Sample	Volume (ml)	Absorbance at 550nm	Dilution	Protein concentration (mg/ml)	Total protein (g)	Fraction purified/ yield (g/g)
Set 1						
Crude extract	35	0.244	200	0.260	1.820	1.000
0-30% saturation	10	0.153	100	0.160	0.160	0.088
30-60% saturation	10	0.287	200	0.305	0.610	0.385
60-90% saturation	10	0.034	100	0.035	0.035	0.019
Set 2						
Crude extract	44	0.244	200	0.260	2.288	1.000
0-60% saturation	10	0.367	200	0.390	0.780	0.341
60-90% saturation	10	0.109	25	0.115	0.029	0.013
Set 3						
Crude extract	34	0.244	200	0.260	1.768	1.000
0-90% saturation	10	0.400	200	0.425	0.850	0.481

Trial 2

Sample	Volume (ml)	Absorbance at 550nm	Dilution	Protein concentration (mg/ml)	Total protein (g)	Fraction purified/ yield (g/g)
Set 1						
Crude extract	35	0.293	200	0.310	2.170	1.000
0-30% saturation	10	0.195	100	0.210	0.210	0.115
30-60% saturation	10	0.332	200	0.350	0.700	0.385
60-90% saturation	10	0.042	100	0.040	0.040	0.022
Set 2						
Crude extract	44	0.293	200	0.310	2.728	1.000
0-60% saturation	10	0.434	200	0.460	0.920	0.337
60-90% saturation	10	0.166	25	0.170	0.043	0.016
Set 3						
Crude extract	34	0.293	200	0.310	2.108	1.000
0-90% saturation	10	0.444	200	0.470	0.940	0.446

Table 2: Ammonium Sulfate precipitation with dialysis

Trial 1

Sample	Volume (ml)	Absorbance at 550nm	Dilution	Protein concentration (mg/ml)	Total protein (mg)	Fraction purified/ yield (g/g)
Set 1						
Crude extract	42	0.130	200	0.140	1.176	1.000
0-30% saturation	12	0.100	100	0.105	0.126	0.107
30-60% saturation	16	0.129	200	0.138	0.442	0.376
60-90% saturation	14	0.002	100	0.005	0.007	0.006
Set 2						
Crude extract	50.6	0.130	200	0.140	1.417	1.000
30-60% saturation	15	0.144	200	0.150	0.450	0.318
60-90% saturation	13.5	0.043	50	0.045	0.030	0.021
Set 3						
Crude extract	39.1	0.130	200	0.140	1.095	1.000
0-90% saturation	16.5	0.115	200	0.120	0.396	0.362

Trial 2

Sample	Volume (ml)	Absorbance at 550nm	Dilution	Protein concentration (mg/ml)	Total protein (mg)	Fraction purified/ yield (g/g)
Set 1						
Crude extract	42	0.127	200	0.135	1.134	1 000
0-30% saturation	12	0.084	100	0.090	0.108	0.114
30-60% saturation	16	0.133	200	0.140	0.448	0.474
60-90% saturation	14	0.010	100	0.010	0.014	0.015
Set 2						
Crude extract	50.6	0.127	200	0.135	1.366	1.000
0-60% saturation	15	0.162	200	0.170	0.510	0.373
60-90% saturation	13.5	0.063	50	0.065	0.044	0.032
Set 3						
Crude extract	39.1	0.127	200	0.135	1.056	1.000
0-90% saturation	16.5	0.133	200	0.140	0.462	0.438

Table 3: Average ammonium sulfate precipitation

Sample	Average fraction purified (yield)	
	without dialysis (g/g)	with dialysis (g/g)
Set 1		
Crude extract	1.000	1.000
0-30% saturation	0.102	0.110
30-60% saturation	0.360	0.425
60-90% saturation	0.021	0.010
Set 2		
Crude extract	1.000	1.000
0-60% saturation	0.339	0.346
60-90% saturation	0.014	0.027
Set 3		
Crude extract	1.000	1.000
0-90% saturation	0.463	0.400

3.2 Effect of the presence of ammonium sulfate on Lowry method

As jacalin concentrations for all trials were obtained based on the Lowry method, the effect of ammonium sulfate on jacalin determination using this method was able to be studied. Even though the yield trends for both with and without dialysis are similar, there is a difference in the amount of yield and also the overall percentage of NH_4SO_4 that would give the maximum yield of jacalin precipitated.

In general, when respective fractions for each set are studied, the values for yield obtained after dialysis was found to be higher than the yield obtained without any dialysis (shown in Table 3). This is caused by the presence of ammonium sulfate in the precipitated extract. Ammonium ions may also interfere with the Lowry reaction [15]. Hence, protein detection using the Lowry method will be less accurate and the absorbance values need to be corrected by considering the presence of ammonium sulfate. However, for the 0-90% saturation, the fraction purified with dialysis is less than that without dialysis. This may be due to the excess ammonium sulfate that is present and the ammonium sulfate may itself being absorbed by the UV wave, thereby increasing the absorbance value at 550nm. From Table 3 (with dialysis), percentage saturation of range 30-60% saturation in set 1 gives the maximum yield, followed by 0-90% saturation in set 3 and 0-60% saturation in set 2. From Table 3 (without dialysis) yield of 0-90% saturation in set 3 is higher compared to 30-60% saturation in set 1. This contradiction is due to the excess of ammonium sulfate salt that causes an increase in absorbance at wave length of 550nm. As bigger portion of jacalin in the crude extract can be precipitated at 60% saturation, further addition of ammonium sulfate salt can only cause a slight increase in jacalin precipitation. After which, an excess amount of salt continue to exist with the precipitated extract and cause the absorbance obtained from the Lowry test to deviate from actual value, consequently it gives higher reading.

The 30-60% saturation range gives the maximum amount of jacalin precipitated. However, 0-90% saturation can also be used. For the remaining portion of this work, the ammonium sulfate 0-90% saturation was chosen to simplify methods.

3.3 Isolation of jacalin by affinity chromatography

3.3.1 Comparison between affinity chromatography purification with and without ammonium sulfate precipitation

From Fig. 1, it can be seen that the peak of absorbance is 0.068 for blank solution containing only 0.2M Galactose and 0.15 M NaCl. On the other hand, the peak absorbance for jacalin elution when only affinity chromatography step is involved in jacalin purification was found to be 0.148 (fraction 31). In order to obtain the reduced peak, the peak profile of blank was subtracted from the original elution peak [18]. Hence, the reduced peak value was 0.08.

For purification with ammonium sulfate precipitation at 0-90% saturation, elution with solution of 0.2 M galactose and 0.15 M NaCl, peak absorbance is 0.260 and the peak absorbance is 0.068 for blank at fraction 23. Hence, the reduced peak is 0.192 (Fig. 2).

Using the calibration curve for absorbance at 280nm, the total amount of protein concentration for both peaks is obtained and presented in Table 4. The table shows that the protein concentration purified via step 2 using both ammonium precipitation and affinity chromatography method yields higher amount of protein compared to step 1 purification, where only affinity chromatography method is used. This is theoretically true especially when precipitation method is combined with the affinity chromatography technique. Researchers in previous works have stated that ammonium sulfate precipitation is considered as a gross fractionation method and usually, it precedes the chromatography methods. The affinity chromatography will then give greater purification [19].

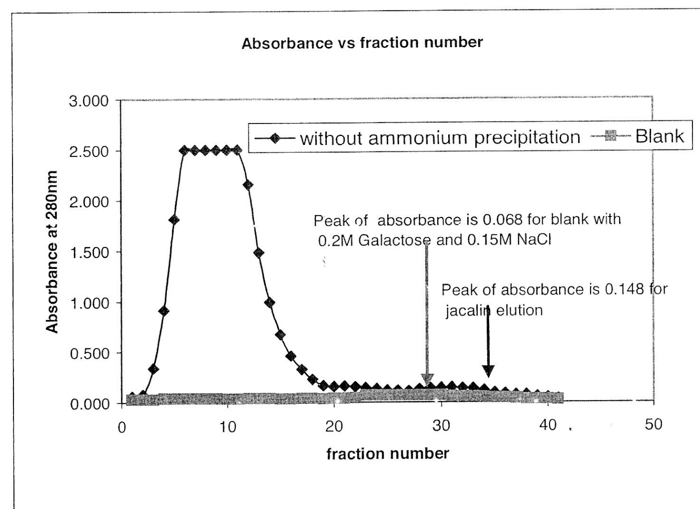

Fig 1: Purification of jacalin from CJ1 by affinity chromatography without ammonium sulfate precipitation

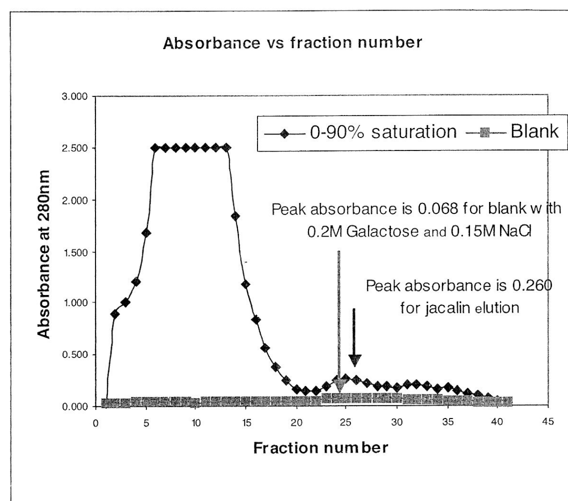

Fig 2: Purification of jacalin from CJ1 by affinity chromatography with 0-90% saturation of ammonium sulfate precipitation and elution with solution of 0.2M galactose and 0.15M NaCl

Table 4: Protein concentration for affinity purification with ammonium sulfate precipitation and without ammonium sulfate precipitation.

Sample	Reduce peak absorbance at 280nm	Protein concentration (mg/ml)
Crude	0.12	0.140
Without ammonium sulfate precipitation, eluted with 0.1M Galactose + 0.15M NaCl	0.08	0.090
0-90% saturation, eluted with 0.1M Galactose + 0.15M NaCl	0.192	0.220

3.3.2 Comparison of elution using different galactose concentrations

Three different concentrations of galactose (in 0.15 M NaCl) ; 0.1 M , 0.2 M and 0.4 M were applied during elution step. From Figure 3, the peak absorbance for blank with 0.1M galactose was 0.065 and the peak absorbance for jacalin elution using 0.1 M galactose was 0.148. Hence reduced peak was calculated to give 0.083. Total protein concentration was 90μg/ml. From Figure 2, the peak absorbance for blank with 0.2 M galactose was 0.068 and the peak absorbance for jacalin elution using 0.2 M galactose was 0.260. Hence the reduced peak was calculated to be 0.192. Total protein concentration was 220μg/ml. From Figure 4, the peak absorbance for blank with 0.4 M galactose was 0.156 and the peak absorbance for jacalin elution using 0.4M galactose was 0.256.

Hence the reduced peak was calculated to be 0.100. Total protein concentration was found to be 110 μg/ml.

Results of the three concentrations of galactose used were compared in Table 5. The table shows that elution using 0.2 M galactose (in 0.15 M NaCl) gives the highest protein concentration followed by elution using 0.4 M galactose and 0.1 M galactose.

The concentration of 0.1 M galactose is capable of eluting jacalin. However, the concentration is not strong enough to fully compete with the galactose concentration coupled to affi-gel. For 0.4M galactose concentration in the carrier stream, it does not show an increase in the amount of protein concentration. On the other hand, a slight decrease in protein concentration is observed. This is due to the conformational changes in the proteins [20] and their molecular structures. No linear correlation can be seen between increased in jacalin concentration and increase in galactose concentration. It appears that concentration below or above 0.2 M will not give the elution of maximum jacalin concentration.

Table 5: Protein concentration using different galactose concentration during elution step on the affinity chromatography (with ammonium sulfate precipitation)

Sample	Reduce peak absorbance at 280nm	Protein (mg/ml)
Crude	0.12	0.140
0-90% saturation, eluted with 0.1M Galactose + 0.15M NaCl	0.083	0.092
0-90% saturation, eluted with 0.2M Galactose + 0.15M NaCl	0.192	0.220
0-90% saturation, eluted with 0.4M Galactose + 0.15M NaCl	0.100	0.110

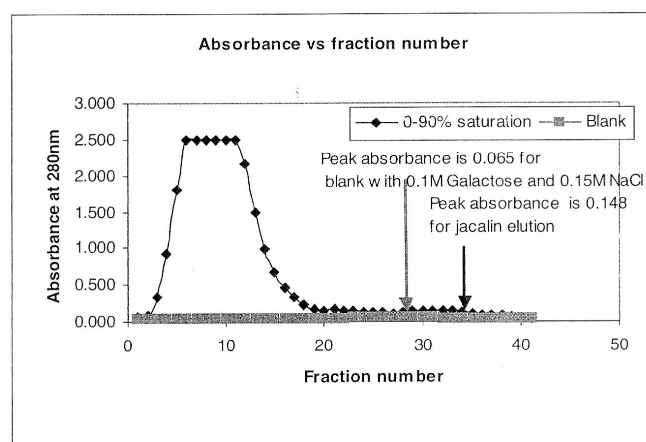

Fig 3: Purification of jacalin from CJ1 by affinity chromatography with 0-90% saturation of ammonium sulfate precipitation and elution with solution of 0.1 M galactose and 0.15 M NaCl

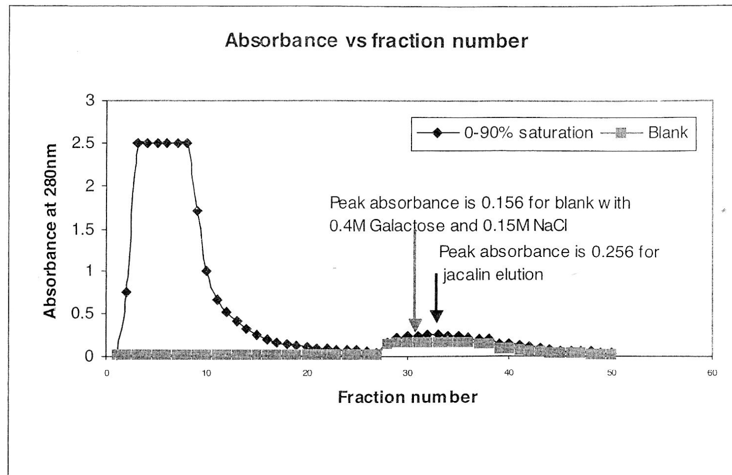

Fig 4: Purification of jacalin from CJ1 by affinity chromatography with 0-90% saturation of ammonium sulfate precipitation and elution with solution of 0.4 M galactose and 0.15 M NaCl

4. Conclusion

Addition of NH_4SO_4 at the 30-60% saturation range gives the maximum amount of protein precipitated followed by 0-90% saturation. However, the 0-90% saturation range is more suitable to be used for large scale applications since it only involves 1 step of NH_4SO_4 addition compared to the 2 step addition to obtain 30-60% saturation. This finding is very useful at deciding which saturation range to be used according to priority of options in scaling up the operation. This study also suggests that dialysis improves the fraction of purified protein. Another important finding is that excess of NH_4SO_4 interferes with Lowry method for protein determination. This is due to the presence of NH_4SO_4 that increases the actual UV spectrophotometer absorbance thereby giving misleadingly higher protein concentration. Jacalin concentration is further increased when both ammonium sulfate precipitation and affinity chromatography steps are involved. Results from this work demonstrate that elution with 0.2 M galactose (in 0.15 M NaCl) gives the best peak profile or the maximum elution of jacalin concentration. Decreasing or increasing galactose concentration beyond 0.2 M does not help in increasing concentration of purified jacalin.

REFERENCES

[1] K. Shahjahan. "The isolation and characterization of jacalin [Artocarpus heterophyllus (Jackfruit) Lectin] based on its charge properties." Int. J. Biochem.Cell Biol., Vol. 27, No. 2, 147-156. 1995.

[2] A. Barre, W.J. Peumans, M. Rossignol, G. Borderies, R. Culerrier, E.J.M. Van Damme and P. Rougé. "Artocarpin is a polyspecific jacalin-related lectin with amonosaccharide preference for mannose." Biochimie, Vol. 86, No. 9-10, 685-691. 2004.

[3] L.L.P. daSilva, J.B. deMolfetta-Machado, A.P. Castelo, J. Denecke, G.H. Goldman, M.C.R. Barreira and M.H.S. Goldman. "cDNA cloning and functional expression of KM+, the