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Extraction and Partial Purification of Thaumatin from Arils of *Thaumatococcus daniellii* Fruit

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Thaumatococcus daniellii is a large flowering herb with rhizomatous rootstock, commonly found mostly in the western part of Nigeria. *Thaumatococcus daniellii* fruit arils that contain the sweetener, are embedded within a gel-like fluid that is richly composed of polysaccharide. The extraction and partial purification of thaumatin (protein) was undertaken to devise simpler means to extract and purify the protein, following the difficulty associated with the extraction due to the presence of the gel that encapsulates the protein. The research was carried out in department of biochemistry laboratory, covenant university Ota, Nigeria, between 10 August 2015 and 13 November 2018. In attempt to determine the optimal pH and temperature for extraction of crude thaumatin in different media from 4,000mg of the sample. Extraction was carried out at different pH values (2, 3, 4 and 5) in varied temperatures (25^{o}C, 30^{o}C, 40^{o}C, 45^{o}C, 50^{o}C, 55^{o}C, 60^{o}C, 70^{o}C, 80^{o}C and 90^{o}C). Maximum concentration of crude thaumatin extracted in 50ml of distilled water was 13.40mg/ml at pH 3 and temperature of 50^{o}C while 28.17mg/ml at pH 3 and temperature of 55^{o}C was extracted in 50ml of 0.85M of aqueous sodium chloride (dilute sodium chloride). Each extracted crude thaumatin was subjected to centrifugation at 10,000rpm for 1800secs, the decanted extracted crude thaumatin (supernatant) formed were assayed for protein via lowry

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method. Having pooled each decanted supernatant, the extracted crude thaumatin were precipitated with ammonium sulphate, dialyzed and subjected to column chromatography while the eluent were collected, tested for protein then freeze dried and stored as partially purified thaumatin. From the above findings, the extraction and partial purification of thaumatin can be optimized in dilute sodium chloride at optimal pH and temperature thereby making protein more readily available. Thaumatin a protein with very low calorie is an excellent alternative to other sweeteners with high calories especially sucrose, consequently will go a long way to reducing health challenges associated with sucrose such as obesity, hyperglycaemia, dental cares and even diabetes.

Keywords: Rhizomatous rootstock; pectinase; supernatant; viscous medium; hyperglycaemia.

1. INTRODUCTION

Thaumatin, a very low calorie intensely sweet-tasty protein [1] (Raimi *et al*), obtained from *Thaumatococcus daniellii (T. daniellii)* fruits, within the fruits it is covered by layer of sticky, transparent gel that has a soft, fleshy and juicy cap called an aril which contain sweet substances (Thaumatin). These arils compose of a gel mainly of polysaccharide origin. These jellies make it very difficult to extract these thaumatin resulting to a low yield during extraction, therefore more scientific effort is needed to increase yield during extraction to make the sweetener readily available as replacement for sugar. The major sweet tasting thaumatin proteins are thaumatin I and thaumatin II along with thaumatin a, thaumatin b and thaumatin c. Thaumatin is by far the sweetest compound known till now [2, 3] (Poldermans and Matt.; Smith), but the major flavor-modifying properties reside in two very similar proteins, thaumatin I and thaumatin II. The thaumatin are first sweet–tasting proteins that have been found in nature and the crystals are about 2,000-3,000 times sweeter than sucrose and neither allergic nor mutagenic or teratogenic [4] (Yeborah *et al*). The arils are attached to the seeds which produce the jelly that swells to many folds its weight and houses the thaumatin. The arils which contain the Thaumatin, constitute 4.8% of the fruit while the fleshy part and the seed account for 72.4% and 22.8%, respectively [1] (Raimi O. *et al*).

Following the high incidence of sugar related diseases associated with the consumption of sucrose, it has turned into the principal driver for the advancement of sugar alternatives that have the improved properties of sucrose yet with little or no calorie. Therefore the urgent need, to replace part if not total while sustaining the same sensation that is derived from sugar, displayed ab initio from the need of sucrose reduction particularly in the diets as a way of reducing sugar in the blood and in the management of conditions associated with high blood sugar especially in diabetics. However, thaumatin having 207-amino acid chain do not cause spikes or rise the blood glucose level, since it possess zero glycaemic index, hence very suitable for diabetics. Therefore Thaumatin a protein with very low calorie is an excellent alternative to sweeteners with high calories mainly sucrose, and will go a long way to reducing health challenges associated with sucrose such as obesity, hyperglycaemia and even diabetes. Irrespective of the numerous uses and advantages of thaumatin in industrial use, thaumatin of plant origin is very limited as a result of the difficulty associated in getting the fruit from which it is extracted [5] (Faus). Although genetic engineering provide an alternative means of thaumatin production in yeast such as kluveromyces lactis, in fungus Aspergillus oryzae, in bacteria such as Streptomyces lividans, and even in transgenenic plants such as Solanum tuberosum just to mention but a few. However, is unbelievable that in spite of these breakthroughs, the availability of the sweetener remain relatively low, very expensive and scarce [6,5] (Kuznetsor et al.; Faus).

2. MATERIALS AND METHODS

2.1 Collection of Samples

The fleshy ripe *Thaumatococcus daniellii* fruits were harvested from *Thaumatococcus daniellii* plant from Otun village bush, Ayetoro local forest in Moba Local Government Area of Ekiti State, South-western Nigeria. The fruits were between the range of 1.4 –2.1cm long, trigonal or pyramidal in shape, deep red and bright red colour as ripe fruits. The weight of fruits were within the range of 10 to 22g depending on the number of seeds inside it. The *Thaumatococcus daniellii* (Benth) fruits were identified by Forest Herbarium, Ibadan 110158. The fruits were

transported to Covenant University, Ota Ogun State, where the fruits were thoroughly washed was initially with tap water and later with distilled water in order to remove any dirt or filth particles present on the surface. Then physically examined and transported to biochemistry laboratory and stored at temperature of -18^{0}C. Four hundred and twenty-two (422) of the fleshy fruits were selected and weighed; the total weight amounted to 6752g (6.752kg). Each of the fruits was peeled, frozen and freeze dried with freeze dryer until the arils became brittle. By hitting the freeze dried fruits with hand repeatedly the brittle aril became separated from seeds and the rest of the fruits. Then the brittle aril was ground using a blender into powdered form. A total weight of 400g of powdered arils was obtained and stored in an air tight bottle as sample for extraction of crude thaumatin.

Sample (Dried Powdered Arils)

Plate: 2.1 *Thaumatococcus daniellii* plants

Plate: 2.2 *Thaumatococcus daniellii* seed, with Arils on it

Plate: 2.3 *Thaumatococcus daniellii* seeds, without Arils on it

2.2 Extraction of Crude Thaumatin in Distilled Water at Different pH Values in Varied Temperatures

Four portions of 40g of the dried powdered sample were weighed, collected and stored separately. Distilled water was also prepared and stored in two separate 1000ml conical flasks each. Each 40mg of the samples was dissolved in 50ml of distilled water, after thorough shaking for 120secs at pH value of 2, 3, 4 and 5 each; 5mls each from the extracted thaumatin were pipetted into 10 empty test tubes. A total of forty test tubes were collected and each was subjected to different temperature of 25^{o}C, 30^{o}C, 40^{o}C, 45^{o}C, 50^{o}C, 55^{o}C, 60^{o}C, 70^{o}C, 80^{o}C and 90^{o}C with the aid of a water-bath adjuster for 1200secs and thaumatin extracted. Then at the end, 4ml from each of the raw crude thaumatin extracted was pipetted into 4ml micro tubes and centrifuged at 10,000rpm for 1,800secs and later tested orally. After the centrifugation (Uniscope Laboratory Centrifuge Surgifriend Medicals, England) each of the supernatant formed was decanted and stored, while little of it was tested orally the remaining portions each were assayed for protein.

2.3 Extraction of Crude Thaumatin in Dilute Sodium Chloride at Different pH Values in Varied Temperatures

After the preparation of exactly 0.85M of aqueous sodium chloride, it was stored in two separate 1000ml of conical flask. Four portions of 40g of the sample were dissolved in 50ml of 0.85M of aqueous sodium chloride, after thorough shaking for 120secs at pH value of 2, 3, 4 and 5 each; 5mls each from the extracted thaumatin were pipetted into 10 empty test tubes. A total of forty test tubes were collected and each

was subjected to different temperature of 25^{o}C, 30^{o}C, 40^{o}C, 45^{o}C, 50^{o}C, 55^{o}C, 60^{o}C, 70^{o}C, 80^{o}C and 90^{o}C with the aid of a water-bath adjuster for 1200secs and thaumatin extracted. Then at the end, 4ml of extracted crude thaumatin each was pipetted into 4ml micro tubes and centrifuged (GEN105 UV-VIs) at 10,000rpm for 1800secs. After the centrifugation each supernatant formed was decanted and assayed for protein via Lowry method, while little of it was tested orally.

2.4 Purification of Crude

2.4.1 Crude thaumatin precipitation and dialysis

The supernatant formed from the various extractions of crude thaumatin was pooled together in two separate 1000ml beakers and freeze dried with the aid of a freeze-dryer to obtain a dried crude thaumatin for purification. Exactly 20g of the freeze dried crude thaumatin crude was dissolved in 200ml of distilled water in a beaker, stirred to dissolve completely, centrifuged at 10,000 rpm for 1800secs and supernatant decanted. At higher salt concentrations, protein solubility decreases, resulting to precipitation, termed salting-out [7] (Green and Hughes), hence 112.2g of ammonium sulphate was added into the decanted supernatant, thoroughly stirred, allowed to stand for 90mins, decanted and transferred into a dialysis bag, tied and placed into a 1000ml beaker containing 700ml volume of water that completely covers the dialysis bag for further purification. A stirrer was placed inside the beaker and mounted on the magnetic stirrer and stirred for four hours. The water inside the beaker was continually changed every 60mins for three consecutive times, while the crude thaumatin was still in dialysis bag.

2.4.2 Fractionation by column chromatography

A glass column was suspended in slanting position with retort stand and glass wool inserted close to upper end of the column. By inserting a stirring glass rod slanting touching the bottom end of the column close to the glass wool while the other end of the stirring glass rod was raised up a bit, then 5g of Sephadex G-25 dissolved with 30ml of distilled water was poured on the upper body via the stirring glass rod. Then a given set (Agary infusion set) filled with water was connected and allowed to be dripping water into the column continuously also via upper end of the column, so as to avoid the gel (Sephadex) to cake (stiff).

Then the dialyzed supernatant (extracted crude thaumatin) were poured in the upper end of the column and the eluent collected with 10ml test tubes. Each test tube was replaced whenever it was filled with the eluent, hence 60 test tubes were used to collect the eluent. Each fraction was subjected to assay using spectrophotometer at 570nm wavelength and the absorbance recorded. Then at the end of the process the fractions were pooled together and subjected to centrifugation, the resultant supernatant were freeze dried, and collected as partially purified Thaumatin and stored in the refrigerator.

3. RESULTS AND DISCUSSION

3.1 Crude Thaumatin Concentrations Extracted in Distilled Water at Different pH Values in varied Temperatures

The concentrations of crude thaumatin in distilled water at varied pH (range; 2.0-5.0) in varied temperature (range; 25^{o}C to 90^{o}C) were determined by means of standard curve, as shown in Fig 1. The calculated values for protein in distilled water are shown in Table 1, the values varied randomly, between 1.74 mg/ml at 25^{o}C and 13.40 mg/ml concentration of thaumatin at 50^{o}C.

3.2 Crude Thaumatin Concentrations Extracted in Dilute Sodium Chloride at Different pH Values in Varied Temperatures

The concentrations of crude thaumatin extracted in dilute sodium chloride from the sample at different pH (range; 2.0-5.0) and in varied temperature (range; 25 to 90^{o}C) were determined also by means of standard plot as shown in Fig 2. The calculated values for protein in dilute Sodium Chloride are shown in Table 2, the values varied randomly, between 4.50 mg/ml at 25^{o}C and 28.17mg/ml concentration of thaumatin at 55^{o}C.

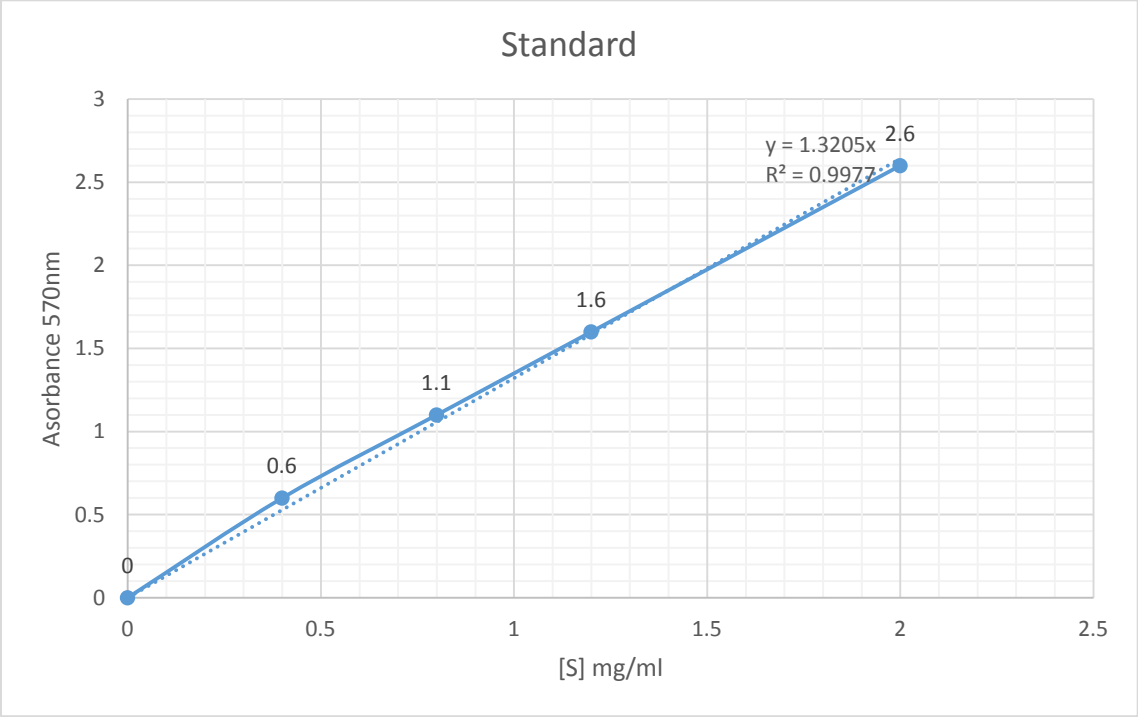

Fig. 1. Lowry standard plots for protein determination in distilled water

Table 1. Crude thaumatin concentration extracted in distilled water at pH values and in varied temperature

Temp (0^oC)	pH 2.0		pH 3.0		pH 4.0		pH 5.0	
	Y (nm)	X (mg/ml)	Y (nm)	X (mg/ml)	Y (nm)	X (mg/ml)	Y (nm)	X (mg/ml)
25	4.3	3.26	5.0	3.80	2.8	2.12	2.30	1.74
30	5.6	4.24	8.5	6.40	4.4	3.33	3.50	2.65
40	12.8	9.70	14.3	10.80	8.1	6.13	7.30	5.53
45	14.4	11.00	16.4	12.40	9.0	6.80	7.70	5.83
50	16.0	12.10	17.7	13.40	14.7	11.13	12.7	9.61
55	14.3	10.83	15.8	12.00	11.3	8.55	10.4	7.90
60	13.7	10.40	14.7	11.00	10.2	7.72	9.80	7.42
70	12.2	9.20	13.2	10.00	8.7	6.60	7.60	5.75
80	11.8	9.00	12.0	9.08	7.4	5.60	6.80	5.20
90	10.3	7.80	11.7	8.86	7.6	5.76	6.70	5.10

Y (Absorbance) =1.3205X
X= Actual conc.

Table 1 above showed that at different pH values, minimal and maximal crude thaumatin were extracted at temperature of 25^oC and 50^oC respectively. At pH 2, crude thaumatin recorded at these temperatures (125^oC and 50^oC) were 3.26mg/ml and 12.10mg/ml, however at pH 3, concentration of 3.80mg/ml and 13.40mg/ml were recorded, and 2.12mg/ml and 11.13mg/ml were recorded at pH 4, while 1.74mg/ml and 9.61mg/ml were recorded at pH 5. From the same table it was also observed that maximum concentration (13.40mg/ml) of crude thaumatin was recorded at pH 3. Therefore the optimal pH and temperature of extraction in distilled water were at pH 3 and 50^oC respectively.

Relatedly, in Table 2 result showed that at different pH values, minimal and maximal crude thaumatin were extracted at temperature of 25^oC and 55^oC respectively. At pH 2, crude thaumatin recorded at these temperatures were 6.70mg/ml and 25.14mg/ml, at pH 3 the concentration of 7.00mg/ml and 28.17mg/ml were recorded, and 5.20mg/ml and 21.35mg/ml were recorded at pH

4 while 4.50mg/ml and 20.14mg/ml were recorded at pH 5. From same the table 2 it was observed that maximum concentration (28.17mg/ml) of crude thaumatin was recorded at pH 3. Therefore the optimal pH and temperature of extraction of thaumatin in dilute sodium chloride were at pH 3 and 55^{o}C respectively.

The lower yield in the concentration of crude thaumatin recorded in distilled water was as a result of the gel that encapsulates the sweet smelling substance (thaumatin) possesses remarkably water-absorbing property/capacity. Therefore when the gel is in contact with water, it swells many times, absorbing up to fifteen times of its own weight of water [8] (Higginbotam) as well as the sweetener (protein) and consequently causes problem in the extraction, since the gel absorbs both sweetener and distilled water (extractant), while in dilute sodium chloride, much higher concentration of crude thaumatin were recorded, suggestive of the water absorbing capacity of the gel was remarkably inhibited [8] (Higginbotham), making the gel to absorb very small amount of the sweetener and the dilute sodium chloride (extractant), hence increases the efficacy, ease crude thaumatin extraction substantially and markedly, therefore this work agrees with similar work carried out

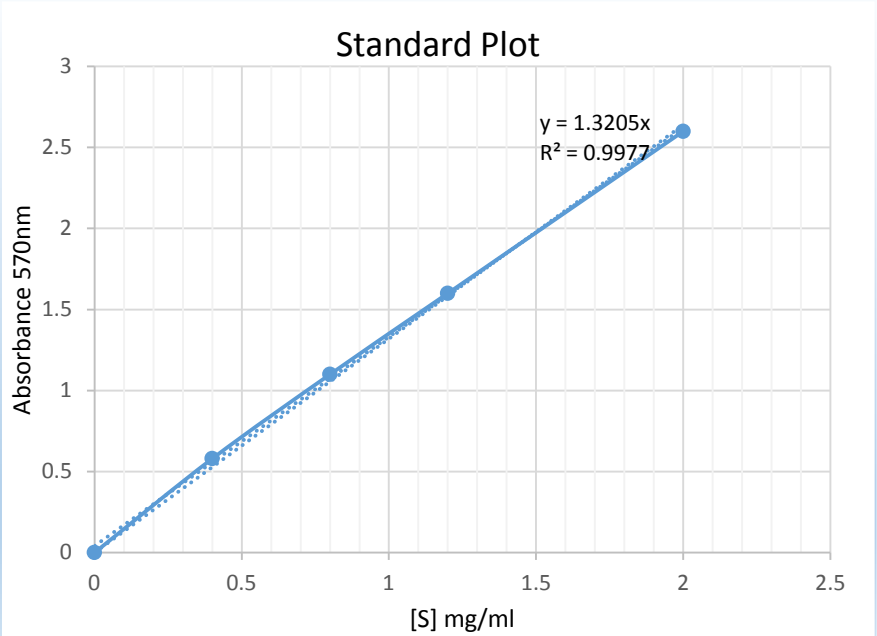

Fig. 2. Lowry standard plots for protein determination in dilute sodium chloride

Table 2. Crude thaumatin concentrations extracted in dilute sodium chloride at different pH and in varied temperature

Temp. (0^{o}C)	pH 2.0		pH 3.0		pH 4.0		pH 5.0	
	Y (nm)	X (Mg/ml)	Y (nm)	X (mg/ml)	Y (nm)	X (mg/mg)	Y (nm)	X (mg/ml)
25	8.8	6.70	9.20	7.00	6.90	5.20	5.9	4.50
30	11.2	8.47	13.2	10.00	8.60	6.50	8.0	6.10
40	20.6	15.60	21.8	16.50	18.2	13.8	16.4	12.4
45	23.0	17.40	27.6	21.00	19.8	15.0	17.4	13.2
50	26.2	19.80	29.1	22.00	23.8	18.0	22.4	17.0
55	33.2	25.14	37.2	28.17	28.2	21.35	26.6	20.14
60	21.4	16.20	23.4	17.70	19.8	15.0	20.0	15.0
70	19.1	14.50	21.8	16.50	17.0	12.8	16.8	13.0
80	16.4	12.40	21.5	16.30	15.6	11.8	14.4	11.0
90	16.2	12.30	21.0	16.00	15.0	11.3	14.2	11.0

Y = Absorbance
Absorbance = 1.3205X,
X= Actual Conc.

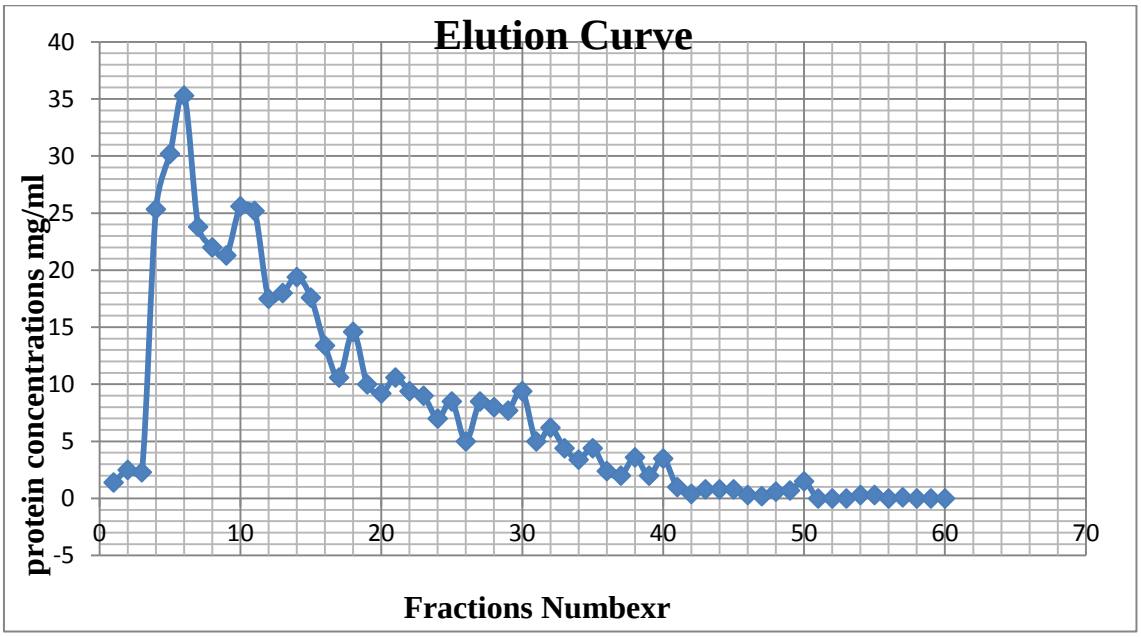

Fig. 3. Thuamatin elution profile

with Higginbotam, 1976. It was also observed that at these pH values in varied temperatures, below 70^{o}C of extraction, the sweetness of the protein was fully retained when tested orally, as opined by [9,10] (Gibbs *et al.*; Lord). However, at 70^{o}C and above this temperature there was slight loss of sweetness which can be attributed to heat breakage or denaturation of disulphide bridges of the protein [11] (Higginbotham). Thaumatin when subjected to boiling in deionised water, thaumatins show very little loss of sweetness even after several hours [11,12] (Higginbotham; Shallenberger).

3.3 Collection of Purified Thaumatin by Chromatography

It was observed that when the ammonium sulphate was added to the dissolved crude thaumatin, the protein precipitates and separates, purification of salt trapped in the protein was removed via dialysis, as the distilled water was removed and replaced intermittently. Further purification of protein was aided by column chromatography, as the protein and solvent moves in the column, the protein separates as it moves down the column. The dissolved sephadex acted as a buffer removed small molecule and aided desalting of the protein even faster. Hence the concentration of contaminants within the crude thaumatin sample was drastically reduced to acceptable/negligible level. Test tubes were set and the eluent collected with sixty test tubes and the absorbance of the thaumatin in each test tube taken. Then subjected to further centrifugation while the resultant supernatants (clear brown liquor) was freeze dried with a freeze-dryer to yield a light, fluffy buff-coloured product, collected as partially purified thaumatin and stored in the refrigerator accordingly. From the Fig. 3 above, it can be seen that large volume of thaumatin were eluted in or just after the void volume of thaumatin as they passed through the column at the same speed as the flow of buffer. The maximum protein concentration elute was 35mg/ml while all through the elution, the curve showed a tortuous curve.

4. CONCLUSION

Thaumatin an excellent alternative sweetener can be viewed to play crucial role as safe for different food item, medicinal, soft drinks etcetera in time to come. The extraction of the intensely sweet juice called thaumatin, is usually very difficult by pressing or via other means in order to extract the sweetener. This is because the aril that houses thaumatin is encapsulated with gel mainly of polysaccharide origin, and these jellied mass is responsible for difficult in the extraction. However by inhibiting the water absorbing capacity of the gel that encapsulate the sweetener with dilute aqueous sodium chloride at pH 3 and temperature of 55^{o}C, higher concentration of crude thaumatin was recorded. Hence the crude thaumatin was precipitated,

dialyzed and subjected to column chromatography and eluent collected as partially purified thaumatin, then freeze dried and stored accordingly. Thaumatin, a sweetener with very low calorie, possesses high potential to ameliorate complications in diabetics unlike carbohydrate related sweeteners that trigger high demand for insulin. Consequently, the extraction and application of thaumatin could be a valuable alternative to high calories sweeteners for diabetics.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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