THAUMATIN RECOVERY VIA BIOENGINEERING ROUTE AND *IN-VITRO* CULTURE OF *THAUMATOCOCCUS DANIELLII*

DEREK CHAN JUINN CHIEH

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THAUMATIN RECOVERY VIA BIOENGINEERING ROUTE AND *IN-VITRO* CULTURE OF *THAUMATOCOCCUS DANIELLII*

by

DEREK CHAN JUINN CHIEH

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In loving memory of my Grandpa

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LIST OF ABBREVIATIONS

2,4-D	Dicholorophenoxyacetic acid
AAD	Absolute average deviation
ADI	Allowable daily intake
ANOVA	Analysis of variance
APS	Ammonium persulfate
ATPS	Aqueous two phase system
BAP	6-Benzylaminopurine
BCA	Bicinchoninic acid
BCIP-NBT	5-bromo-4-chloro-3-indolylphosphatetouidinium – nitroblue tetrazolium
BSA	Bovine Serum Albumin
CCD	Central composite design
CCRD	Central composite rotatable design
cp	Number of center points
Da	Dalton
DF	Degree of freedom
DT	Doubling time
DMSO	Dimethyl sulfoxide
DOE	Design of experiment
DTT	Dithiothreitol
DW	Fresh weight
EHEC	Ethyl hydroxyl ethyl cellulose
ELISA	Enzyme Linked Immunosorbent Assay
EU	European Union
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FEMA	Flavor Extract Manufacturers Associations

FW	Fresh weight
GRAS	Generally Regarded As Safe
HPLC	High Performance Liquid Chromatography
HPS	Hydroxypropyl starch
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IgG	Immunoglobulin gamma
JECFA	Joint WHO/FAO Expert Committee on Food Additives
JH III	Juvenile Hormone III
LSD	Least significant difference
MPD	2-Methyl-2,4-pentanediol
MS	Murashige and Skoog
NAA	Napthaleneacetic acid
OFAT	One Factor At a Time
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline with Tween 20
PEG	Polyethylene glycol
pI	Isoelectric point
PKU	Phenylketonuria
RNA	Ribonucleic acid
rpm	revolution per minute
RSM	Response Surface Methodology
SDS	Sodium dodecyl sulfate
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Salt extract
SSE	Error sum of squares
SST	Total sum of squares

TDZ	Thiadiazuron
TL	Tie line
TMA	Taste modifying action
TMP	Taste modifying protein
UK	United Kingdom
UV	Ultraviolet
WE	Water extract
WHO	World Health Organization

LIST OF SYMBOLS

C_t	Top phase concentration (g/ml)
C_b	Bottom phase concentration (g/ml)
DW_f	Final dry weight (g)
DW_i	Initial dry weight (g)
k	number of factors
K	Partition coefficient (dimensionless), define by $K = C_t/C_b$
M	Molar (mol/L)
n	Replicate
p	Number of experimental runs
p	Fractionalization elements
R	Ratio of top phase volume to the bottom phase volume (dimensionless), define by $R = V_t/V_b$
R^2	Coefficient of determination (dimensionless), define by $R^2 = 1 - (SSE/SST)$
r_i	Residual
S	Sugar consumed (g sugar)
T	Duration (day)
V_t	Top phase volume (ml)
V_b	Bottom phase volume (ml)
X	Coded variable
x_{max}	Maximum value of a variable
x_{min}	Minimum value of a variable
Y	Yield (%)
$Y_{x/s}$	Observed biomass yield (dimensionless), define by $Y_{x/s} = (DW_f - DW_i)/S$
y_{exp}	Experimental value
y_{cal}	Predicted value

Greek letters

α Alpha value

σ^2 Variance

σ_{sm}^2 Variance of sample mean

ρ_t Top phase density (g/ml)

ρ_b Bottom phase density (g/ml)

μ Specific growth rate (day^{-1}), define by $\mu = (DW_f - DW_i)/S*T$

PEROLEHAN THAUMATIN SECARA BIOKEJURUTERAAN DAN KULTUR *IN-VITRO THAUMATOCOCCUS DANIELLII*

ABSTRAK

Peningkatan dalam permintaan untuk ramuan pemanis asli telah menarik minat para penyelidik dan saintis kepada protein perisa manis. Sehingga kini, tujuh protein telah dikenalpasti berupaya memberi rasa manis kepada manusia. Salah satu daripadanya adalah thaumatin iaitu protein perisa manis dari tumbuhan *Thaumatococcus daniellii*. Namun begitu, sumber bekalan yang terhad dan kos pemprosesan yang tinggi telah menghalang penggunaannya secara meluas. Kaedah penyarian yang menggunakan garam aluminium menyebabkan produk akhir mengandungi garam tersebut. Walaupun ia boleh diabaikan, namun ia tidak begitu diterima luas oleh pengguna. Kajian untuk pengeluaran secara besar-besaran thaumatin dengan menggunakan perumah rekombinan masih lagi tidak mencapai tahap yang ekonomikal. Oleh itu, kajian ini dijalankan untuk membangunkan protokol penyarian berasaskan air dengan menggunakan kaedah lazim dan juga sistem dua fasa berair (ATPS) yang mempunyai potensi aplikasi dalam industri. Kaedah penyarian dan penulenan lazim terhadap kedua-dua sarian air dan sarian garam dari buah *T. daniellii* memberi keputusan yang berbeza. Ketulenan yang tinggi iaitu 92.4 % telah dicapai untuk sarian air tetapi sarian garam adalah hanya pada 60.2 %. Penghasilan thaumatin untuk sarian air pada 67.4 %, adalah lebih rendah berbanding kepada sarian garam pada 78.2 %. Protokol penyarian dan penulenan yang dibangunkan didapati lebih sesuai untuk sarian air tetapi perlu diulang kaji semula sekiranya hendak diaplikasikan terhadap sarian garam. Prestasi dua sistem ATPS yang terdiri daripada polietilena glikol (PEG)/kalium fosfat dan PEG/natrium sulfat telah dikaji menggunakan rekabentuk faktoran pecahan. Keputusan kajian menunjukkan bahawa sistem PEG/natrium sulfat adalah lebih baik berbanding dengan sistem PEG/kalium fosfat dan PEG, natrium sulfat dan natrium klorida dikenalpasti sebagai

faktor utama dalam pemetakan thaumatin. Pengoptimuman dengan kaedah permukaan sambutan (RSM) terhadap pekali pemetakan thaumatin menghasilkan sistem dengan PEG pada kepekatan 5.92 % (wt/wt), natrium sulfat pada 19.54 % (wt/wt) dengan 0.95 M natrium klorida pada pH 7 dan nisbah isipadu 0.12. Keadaan optimum tersebut memberi penghasilan thaumatin yang tinggi (dari 87 % ke 94 %) apabila diterapkan kepada kedua-dua sarian air dan sarian garam. Keputusan ujikaji menunjukkan bahawa ATPS yang dibangunkan mempunyai potensi untuk digunakan di peringkat permulaan penyarian dan penulenan thaumatin dan juga untuk pengurangan isipadu sebelum proses penulenan seterusnya.

Kajian kultur *in-vitro* pula menunjukkan bahawa kalus dapat diperolehi daripada eksplan akar *T. daniellii* yang dikultur dalam media Murashige dan Skoog (MS) dengan 0.5 mg/L 2,4-diklorofenoksi asid asetik (2,4-D). Kajian yang lebih mendalam diperlukan untuk biosintesis thaumatin secara *in-vitro*. Pertumbuhan sel *T. daniellii* yang lambat telah menghadkan kajian selanjutnya untuk penghasilan thaumatin daripada kultur sel ampaiannya. Oleh yang demikian, integrasi sistem penurasan membran dalam kultur sel tumbuhan telah dijalankan terhadap *Cyperus aromaticus* sebagai sel model. Pengisian semula media melalui sistem membran terhadap sel model memberi biojisim sel dan penghasilan metabolit yang lebih tinggi dengan peningkatan masing-masing sebanyak 64 % dan 112 % pada masa pengkulturan yang sama dengan kumpulan kawalan. Pengurangan dalam tempoh penggandaan, penambahan tempoh pertumbuhan eksponen dan peningkatan dalam kadar pertumbuhan tentu juga diperhatikan dalam kultur yang mengalami pengisian semula media. Pengisian semula media pada 50 % (v/v) telah dikenalpasti boleh laksana dan ekonomikal untuk diaplikasikan dalam skala industri untuk penghasilan hormon remaja (JH) III daripada kultur ampaian *C. aromaticus*. Aplikasi membran turasan pada kultur sel ampaian *T. daniellii* menggunakan data yang diperolehi daripada sel model tidak menunjukkan perbezaan yang bererti. Media pertumbuhan yang lebih sesuai perlu dibangunkan sebelum sistem penurasan membran secara *in-situ* dapat dimanfaatkan.

THAUMATIN RECOVERY VIA BIOENGINEERING ROUTE AND *IN-VITRO* CULTURE *OF THAUMATOCOCCUS DANIELLII*

ABSTRACT

Sweet tasting proteins have sparked new interest amongst researchers and scientist alike due to the increased demand for natural sweetening ingredients. To date, there are seven proteins identified to be able to elicit sweetness in humans. One of them is thaumatin, a sweet tasting protein from the plant of *Thaumatococcus daniellii*. Unfortunately, the limited supply and high processing cost has prevented its widespread use. The current extraction method employs aluminium salt which is present in the final product. Though considered negligible, it is not well accepted by the consumers. Attempts to mass produce thaumatin using recombinant host is yet to achieve an economically feasible level. Therefore this study was carried out to establish a water based extraction protocol for thaumatin using the conventional method as well as aqueous two phase system (ATPS) for potential industrial scale application. The *in-vitro* culture study with the integration of membrane filtration system was also carried as an alternative for thaumatin production in industrial scale. The conventional extraction and purification method employed on both the water and salt extracts of *T. daniellii* fruits showed varying results. A high purity of 92.4 % was achieved for the water extract while the salt extract was only at 60.2%. The yield of thaumatin was somewhat lower for water extract, 67.4 % compared to the salt extract at 78.2 %. The developed extraction and purification protocols were found to be suitable for water extracts but required further revisions in order to be applied to the salt extracts. The performance of two ATPS composed of polyethylene glycol (PEG)/potassium phosphate and PEG/sodium sulfate were evaluated with the aid of fractional factorial design. The study showed that PEG/sodium sulfate system was superior to PEG/potassium phosphate system and consequently, PEG, sodium sulfate

and sodium chloride were identified as the significant factors against thaumatin partitioning. Optimization carried out with the aid of response surface methodology (RSM) with respect to thaumatin partitioning coefficient lead to a system with PEG at the concentration of 5.92 % (wt/wt) PEG, 19.54 % (wt/wt) sodium sulfate with 0.95 M sodium chloride at pH 7 and volume ratio of 0.12. The optimized condition led to a relatively high yield (from 87 % to 94 %) of thaumatin when applied to both the crude water and salt extracts. The results indicated that the ATPS developed showed potential to serve as initial extraction and purification step as well as volume reduction for subsequent purification procedure.

The *in-vitro* culture studies on the other hand showed that callus could be obtained from root explants of *T. daniellii* cultured on Murashige and Skoog (MS) medium supplemented with 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). Detailed studies would be required for possible *in-vitro* biosynthesis of thaumatin. The slow cell growth of *T. daniellii* limited further studies on the production of thaumatin from its cell suspension cultures. The integration of membrane filtration system in plant cell culture was then carried out on *Cyperus aromaticus,* as model cell. Medium replenishment through the membrane system leads to higher cell biomass and metabolite production from the model cells which correlate to a value as high as 64 % and 112 %, respectively, under the similar cultivation period as the control group. The reduction in doubling time, extended exponential growth and increment in specific growth rate were also demonstrated in culture undergoing medium replenishment. The medium replenishment at 50 % (v/v) was identified for an economical and feasible application in industrial scale for juvenile hormone (JH) III production from *C. aromaticus* cells. Application of membrane filtration unit on the cell suspension culture of *T. daniellii* using the data obtained from the model cell however was shown not to differ significantly. Establishment of a more suitable proliferation medium formulation would be required before the incorporation of *in-situ* membrane filtration unit could be benefited.

CHAPTER 1

INTRODUCTION

1.1 Introduction

The appreciation of the sensation of sweetness runs deep in the human psyche. The sweet tasting food has always been associated with food which is safe and palatable to eat. Human craving for sweet food however have lead to health risk such as dental decay, diabetes and obesity. In response to reduce the consumption of sugar, many artificial sweeteners have been developed. Artificial sweeteners not only are able to mimic the effect of sugar but also with much lower calorie content. The most popular class of artificial sweeteners are the high intensity sweeteners. These compounds, sometimes also known as low calorie sweetener, have the sweetness of few hundred to thousand times than that of sucrose but the calorie contribution is often negligible since it is used in minute quantities. In the United States, five artificially derived sugar substitutes have been approved for consumption, namely, saccharin, aspartame, sucralose, neotame and acesulfame K (Sugar substitute, 2007). The usage of the sweeteners has been approved by the US Food and Drug Administration (FDA) but with controversies hanging over them. The ongoing controversies over the supposed health risks free of artificial sweeteners can be found documented elsewhere. The public is more interested in a natural sugar substitute than the artificially synthesized compounds.

Functional bio-proteins, thaumatin from *Thaumatococcus danielli* has sparked new interest amongst researchers and scientist alike due to increased demand for natural sweetening ingredients. Thaumatin, a sweet protein, is 3000 times sweeter than sugar on weight basis (De Vos *et. al.*, 1985). The West African natives have been using the fruits for centuries to sweeten their bread, tea and palm wine (Gibbs *et al.*, 1996). The stability of thaumatin under acidic conditions and at high temperatures as well as pasteurization conditions makes it a very attractive sweetener in food and

pharmaceutical application. Below the threshold of sweetness, thaumatin can also contribute to other complex attributes such as flavour enhancement and masking astringency and off-flavours especially in medicines. As a result, thaumatin can be used as a sweetener, a taste modifier or taste enhancer depending on the type of food and the application.

A successful thaumatin extraction process from the fruits of *T. danielli* has been developed however the presence of aluminium in the final product, though considered negligible, does not bring an attractive image among the consumers (Witty, 1998). The limited supply of fruit and high processing cost has also prevented their widespread use. Therefore, many attempts of expressing thaumatin in recombinant host have been initiated, but so far none is yet to be able to achieve an economically feasible expression level.

The study here is aimed not only on the alternative extraction method for thaumatin but also the production of it through plant cell culture with the incorporation of membrane technology. A conventional water based extraction method using salt precipitation and chromatography was studied and the efficiency and yield of thaumatin was evaluated. An aqueous two phase system (ATPS) could be a useful protein fractionation technique as it is mild in treatment to ensure protein functionality. The integration of ATPS into the purification steps of thaumatin from the fruits offer a reduction and shortening of the conventional purification steps. The development of ATPS could also serve as an excellent tool for the fractionation of thaumatin from the plant cell suspension culture to isolate the cell debris from liquid broth after homogenization. A statistically designed experiment (fractional factorial design) will be used to efficiently screen out the few important main factors from the many less important ones. Response surface methodology (RSM) experimental design will then be applied to the significant factors for the estimation of interaction and locate the optimal process setting for thaumatin purification process.

As for thaumatin production, plant tissue culture may be considered as potential means of producing the taste modifying protein (TMP) in a factory setting as well as a source of them. Plant tissue culture might be able to ensure a continuous supply of uniform quality, highly specialized natural compounds that cannot be produced in equal quality or specificity by other means of biotechnological production. In addition to that, it also offers the possibility of quality control and availability independent of environmental changes. Numerous functional bio-products have been successfully produced through this technology.

The integration of membrane system into the plant suspension culture would provide *in-situ* separation through the natural separation depending on size without any chemical changes. System integrating *in-situ* membrane separation has been shown to have a higher biomass density due to the continuous removal of spent medium and the replenishment with fresh medium (Kamoshita *et al.*, 1998a, Kamoshita *et al.*, 1998b). Harmful secreted product can also be removed in the process and thus enhancing cell viability. The feeding of nutrient is also shown to be very promising and useful technique in increasing metabolite productivity in plant cell culture (Eddie *et. al.*, 2002; Zhang and Zhong, 1997, Zhong and Yoshida, 1995). It is envisaged that such set up could be applied to enhance the yield of thaumatin production as well as valuable metabolite in other plant cell culture in general.

Hence, the study wishes to achieve the following objectives:

1. To develop the appropriate extraction and purification method of thaumatin based on conventional (salting out and chromatographic method) laboratory scale protocol.
2. To develop optimization routine for thaumatin extraction and purification using aqueous two phase system (ATPS).
3. To compare the performance of ATPS with the conventional method

4. To develop *in-vitro* cell culture system for the production of the thaumatin from *T. daniellii* cell suspension cultures
5. To develop an appropriate membrane filtration system for improvement of cell growth and metabolite production in plant cell suspension culture.

CHAPTER 2

LITERATURE SURVEY

2.1 Sweetening agent

Sugar has been traditionally the most popular sweetening agent in food industry. Table sugar or sucrose is the most commonly used to sweeten food stuff and beverages. There are also other types of sugar which tastes sweet such as glucose and fructose, which can be found in fruits and lactose in milk. These sugars, which are carbohydrates, provide approximately 4 calories per gram but they differ in their degree in sweetness. If the relative sweetness of sucrose is given as 1.00, then sweetness of glucose would be 1.45 times of sucrose and that of fructose, the sweetest of all sugar, is 1.65 (Bruice, 1998).

Although sugar gives palatable and pleasant taste to food, the high intake of these sugars has been associated with health risk such as obesity, heart disease and dental decay. Sugar is then substituted with artificial sweeteners to lower its content in food. Artificial sweeteners duplicate the effect of sugar but often with fewer calories. An important class of artificial sweeteners is the high intensity sweeteners. These compounds have the sweetness of many times that of sucrose but the calorie contribution is often negligible since it is used in minute quantities and hence they are sometimes called low calorie sweetener. In United States, five artificially derived sugar substitutes have been approved for consumption. They are saccharin, aspartame, sucralose, neotame and acesulfame K.

Saccharin, the first synthetic sweetener was discovered by accident by Ramsen and Fahlberg in 1879. It is 300 times sweeter than glucose and can be called a true 'non-calorie' compound as it is eliminated by the body intestinal tract without undergoing chemical degradation or metabolism. It has a bitter aftertaste which can be minimize by mixing it with other sweeteners. Saccharin is often used to improve the taste of toothpaste, dietary food and dietary beverages (Bruice, 1998). Aspartame, a

dipeptide from aspartic acid and phenylalanine, was discovered by chance by Schatter in 1965. Aspartame is 200 times sweeter than sugar and is used as tabletop sweetener, frozen desserts, beverages and chewing gum. Though it has no bitter aftertaste like saccharin, its drawback is that it only maintains its sweetness between pH 4 and 8 and is not suitable for baking or other applications requiring extended periods of high temperature (Gibbs *et al.*, 1996). It is by far the most popular sweetening agent but however is not suitable for phenylketonurea (PKU) sufferers. Sucralose is a modified sugar compound which is about 600 times as sweet as sugar. It was produced when 3 chlorine atoms replace 3 hydroxyl groups in a sucrose molecule. It was discovered in 1967 by a chemist through the confusion by 'tasting' it instead of 'testing' it. Sucralose can be used in beverages, frozen desserts and gum. It is also stable in heat and can be used in baking and fried foods. Sucralose is minimally absorbed by the body and passed out unchanged. Neotame is an artificial sweetener similar to aspartame but with a 3,3-dimethylbutyl group attaching to the amino group of aspartic acid portion of the molecule. The attached group prevents the breakage of the peptide bond and reduces the availability of phenylalanine. Therefore it is considered safe for PKU sufferers. Neotame is 8,000 times as sweet as sugar and is moderately heat stable. It has just been recently approved for general use and is a relatively new artificial sweetener. Acesulfame K or also known as acesulfame potassium is 200 times sweeter than sugar but it has slightly bitter after taste especially at high concentration. Unlike aspartame, acesulfame K is stable under heat, under moderately acidic or basic condition. The heat stability of acesulfame K allows it to be used in baking and in products that require a long shelf life.

All the above sweeteners have been approved by the US Food and Drug Administration (FDA) but with controversies surrounding them. The ongoing controversies over the supposed health risks of artificial sweeteners can be found all over the internet. Many people are now looking for natural sugar substitute rather than the artificially synthesized compounds.

2.1.1 Taste modifying protein (TMP)

Some amino acids are known to be mildly sweet while others are perceived as both sweet and bitter. Alanine, glycine and serine are among the sweetest. Large molecules of protein however generally do not stimulate taste cells and hence have no taste. However, there are limited numbers of proteins which have been discovered that are able to stimulate the taste receptors. The existence of these sweet tasting proteins in nature has been known to man for many years. All these proteins have been found in the fruits of tropical plants and the indigenous peoples have frequently used them to sweeten their food stuffs. To date, six sweet proteins that educe intense sweetness have been discovered. They are thaumatin, monellin, mabinlin, brazzein, pentadin and curculin. The seventh protein, miraculin, is by itself not a sweet tasting protein but rather a taste modifier. Table 2.1 and Table 2.2 show the source and a comparison of their properties respectively.

Thaumatin (Van der Wel *et al.*, 1975) and monellin (Gibbs *et al.*, 1996) are the two very well characterized sweet tasting proteins. Thaumatin will be discussed in detail in the following section. Monellin, extracted from the fruits of *Dioscoreophyllum cumminsii* is about 3000 times sweeter than sucrose on a weight basis (Morris and Cagan, 1972). It consist of two polypeptides of 45 and 50 amino acid residues which are associated via non-covalent interactions and gives a total molecular weight of 11 086 Da (Kohmura *et al.*,1990). It can maintain its sweetness between pH 2.4 and 9.6 but loses it taste at pH 10 due to unfolding of its tertiary structure. However its activity is restored upon acidification. The sweetness profile of monellin is a little unusual. The sweetness is not detected for the first few seconds and gradual increase occurs and followed by a slow decline for up to an hour. Mabinlin is obtained from the seed of *Capparis masaikai*, a plant found in South China (Liu *et al.*, 1993). There are five homologous mabinlin which have been isolated and the most studied of them all is designated mabinlin II. Mabinlin II is found to be 375 times sweeter than sucrose and is extremely heat stable (Liu *et al.*, 1993). It is also the most abundant protein found in

Table 2.1: Sweet proteins

Protein	Group	Structure	Natural sources	Reference
Thaumatin	1	22 kD monomer	*Thaumatococcus daniellii* Benth. (West Africa) Marantaceae	Van der Wel and Loeve,1972
Monellin	1	11 kD heterodimer	*Dioscoreophyllum cumminsii* Diels. (West Africa) Menispermaceae	Morris and Cagan, 1972
Mabinlin	1	12.4 kD heterodimer	*Capparis masaikai* Levl. (Yunnan, China) Capparaceae	Liu *et al*., 1993
Pentadin	1	Unknown	*Peneadiplandra brazzeana* Baillon. (Tropical Africa) Pentadiplandraceae	Van der Wel *et al*., 1989
Brazzein	1	6.4 kD monomer	*Peneadiplandra brazzeana* Baillon. (Tropical Africa) Pentadiplandraceae	Ming and Hellekant, 1994
Miraculin	2	24.6 kD homotetramer	*Richardella dulcifica*. (West Africa) Sapotaceae	Theerasilp and Kurihara, 1988
Curculin	2	27.8 kD homodimer	*Curculigo latifolia*. (Western Malaysia) Amarcyllaceae	Yamashita *et al*., 1990

Note: Group 1 proteins are sweet tasting at concentrations as low as 8^{-10} M
Group 2 proteins modify perception of taste and cause sour substances to be perceived as sweet.

Table 2.2: Sweet proteins and their properties

	Thaumatin	Monellin	Mabinlin (II)	Brazzein	Pentadin	Miraculin	Curculin
Found	1972	1972	1986	1994	1989	1960s	1990
Sweetnes factor (M basis)	x 100, 000	x 100, 000	x 375	?	**?**	x 400, 000	x 20, 000/9K
Sweetnes factor (W basis)	x 3, 000	x thousand	X 100	X 2,000	x 500		x 550
Amino acid	207 (exception of 5 residues)	Chain A – 44/45 Chain B – 50	Chain A – 33 Chain B – 72	?	54 monomer	191 tetradimer	114 homodimer
Molecular weight (Da)	T I (22, 209) T II (22,293)	11,086 (A-5251, B-5835)	14, 000	6.5	12, 000	28, 000	27, 800
Isoelectric pont (pI)	12.0	9.0 – 9.4	11.3	?	?	9.1	7.1
Stability	Stable at pH < 5.5 T > 100 °C Pasteur able	Stable at 2.4< pH <9.6 not at pH>10 Stability limited	100°C for 48 hrs (II) 80°C for 1 hr (III,IV)	Sweetness retain even after 80°c for 4 hrs	?	Very stable (pH 4 at 5° for 6 months)	TMA unchanged when incubated at 50 °C for 1 hr between pH 3–11
Sweetness profile	Relatively slow. Liquorice aftertaste	Sweetness not detected for few second, gradually increases followed by slow decline	Similar to sucrose	?	Sweetness increase slowly and declined like monellin and thaumatin	Tasteless but have TMA	Similar to sucrose and have TMA

the seed (1.4 g from 100 g of seeds). The molecular weight of the protein is estimated at 14 kDa and the isolelectric point at 11.3. Brazzein and pentadin are isolated from the fruit of a similar plant, *Pentadiplandra brazzeana*, a climbing shrub found in some countries of tropical Africa. Pentadin, a 12 kDa protein, is 500 times sweeter than sucrose on weight basis and have a similar sweet tasting profile as monellin and thaumatin (Van der Wel *et al.*, 1989). Brazzein on the other hand has a molecular mass of 6473 and like thaumatin, it is a single chain protein with 54 amino acids (Ming and Hellekant, 1994). As pentadin has not been sequenced, it can not be ruled out that there are possibilities that brazzein might be homologous to pentadin and that the two proteins might be related or belong to the same family. There are, however another type of proteins that stimulate taste receptors in taste-modifying fashion. One of them is miraculin a 28 kDa glycoprotein from the berries of *Richadella dulcifica*. Miraculin has the unusual property of modifying sour taste into a sweet taste though miraculin itself has no sweet taste (Theerasilp and Kurihara, 1988). A maximum value of sweetness after exposure to 0.1 mM of miraculin induced by 0.1 M of citric acid was equal to 400,000 times that of sucrose. The second one, curculin isolated from the fruits of *Curculigo latifolia*, not only has the taste-modifying activities but also elicits a sweet taste at the same time (Yamashita *et al.*, 1990). Curculin, a protein with 114 amino acid residues, is 550 times sweeter than sucrose on sugar basis. Curculin has a remarkable ability of turning sour taste into sweet ones. In addition to that, after consuming the fruits, water will elicits a sweet taste and black tea tastes sweet without sugar. It is the only known protein to have both sweetness and taste modifying properties.

2.1.2 *Thaumatococcus daniellii* plant

The scientific name of the plant '*Thaumatococcus*' actually means "fruit of wonder" and according to Witty (1998) the word '*daniellii*' pays tribute to an amateur botanist, William Daniell, who described it about 150 years ago. It grows in the rain

forest areas of West Africa, predominantly in Sierra Leone to Zaire. It is also known to exist in the Princes Islands, Angola, the Central African Republic, Uganda and Indonesia.

Katemfe, a local name for *T. daniellii*, is rhizomatous, perennial and monocotyledonous herb propagation itself by rhizomes. About 2 or 2.5 m long petioles arise from the rhizomes depending on the age and the environment of the plant (Plate 2.1 (A)). At the end of these long petioles are large broad and oval papery, tough, versatile leaves that are about 45 cm long and 30 cm broad. The leaves are ovate-elliptic rounded, truncate at the base and shortly acuminate at the apex. The leaves are used to wrap products to be taken to market or food to be boiled. The leaves together with the petioles are used for thatching and weaving baskets and mats and the petiole pith can be compressed into paper (Witty, 1990).

The inflorescence of katemfe usually arises from the lowest node and may be simple or forked with spikes about 8 to 10 cm in length and bracts, usually umbricate, about 3 to 4 cm in length. The flowers that may be as long as the bracts form in short spikes close to the ground at the base of the swollen petiole (Plate 2.1 (B)). As many as 10 to 12 purple pinkish flowers may arise on each inflorescence but usually only 2, 3, 4 or rarely more than 4 of these form matured fruits (Plate 2.1 (C)). The plant flowers most of the year but is most prolific from July until late October, followed by fruit formation, maturing and ripening from January until mid April. The West African natives have been using the fruits for centuries to sweeten bread, tea and palm wine (Gibbs *et al.*, 1996).

Plate 2.1: (A) *Thaumatococcus daniellii* plant (B) Inflorescence of *Thaumatococcus daniellii* (C) Matured fruit of *Thaumatococcus daniellii*

2.1.3 Thaumatins

The thaumatins are a family of very sweet proteins present in the fruits of the tropical plant *Thaumatococcus daniellii* Benth. The sweet tasting component of *T. daniellii* Benth was identified as protein by van der Wel and Loeve (1972) who were first to isolate them. There are two major form of thaumatin extracted from the fruit of *T. danielli.* They are designated as thaumatin I and thaumatin II. Both have similar properties such as amino acid composition, sweetness profile and identical amino acids sequence except that they differ from one another by only 5 amino acids residues (Figure 2.1) (Faus and Sisniega, 2003). There are no histidine residues in both the thaumatin but there are eight disulfide bonds in the molecule (De Vos *et al.*, 1985).

The molecular weight of thaumatin I and thaumatin II, which are 22,209 Da and 22,293 Da respectively, are very close with one another. X-ray crystallography on thaumatin I crystal showed that thaumatin I consists of 207 amino acids in two building motifs: a folded β sheet or a flattened β 'barrel' and the β ribbons and small loops stabilized by disulfide bonds (Figure 2.2 (A) and (B)) (De Vos *et al.*, 1985). It is also postulated that thaumatin II adopts the similar three dimensional structures as thaumatin I as they differ only by five different amino acids.

Thaumatin is found to accumulate up to 50 % of the dry weight of the aril of the fruit and not be found in any other parts of the plant (Witty, 1990). The protein is extremely soluble in water but not in organic solvent. Thaumatin is stable even at pH below 5.5 and may be boiled for over an hour and regain its sweetness upon cooling. It is also stable under pasteurization conditions. The stability of the protein is due to the presence of eight disulfide bonds in the molecules (Witty, 1990). Thaumatin, with many basic groups in the protein structure, has an isoelectric point (pI) of 12. Thaumatin has been identified to be 100,000 times sweeter than sugar on a molar basis and 3 000 times on a weight basis (De Vos *et. al.*, 1985).

```
ThaumatinI     1 ATFEIVNRCSYTVWAAASKGDAALDAGGRQLNSGESWTINVEPGTNGGKIWARTDCYFDD
ThaumatinII    1 ATFEIVNRCSYTVWAAASKGDAALDAGGRQLNSGESWTINVEPGTKGGKIWARTDCYFDD
                 ********************************************* **************

ThaumatinI    61 SGSGICKTGDCGGLLRCKRFGRPPTTLAEFSLNQYGKDYIDISNIKGFNVPMNFSPTTRG
ThaumatinII   61 SGRGICRTGDCGGLLQCKRFGRPPTTLAEFSLNQYGKDYIDISNIKGFNVPMDFSPTTRG
                 ** *** ******** ************************************ *******

ThaumatinI   121 CRGVRCAADIVGQCPAKLKAPGGGCNDACTVFQTSEYCCTTGKCGPTEYSRFFKRLCPDA
ThaumatinII  121 CRGVRCAADIVGQCPAKLKAPGGGCNDACTVFQTSEYCCTTGKCGPTEYSRFFKRLCPDA
                 ************************************************************

ThaumatinI   181 FSYVLDKPTTVTCPGSSNYRVTFCPTA
ThaumatinII  181 FSYVLDKPTTVTCPGSSNYRVTFCPTA
                 ***************************
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Figure 2.1: Primary structure of thaumatin I and thaumatin II with five different amino acids highlighted (Faus and Sisniega, 2003)

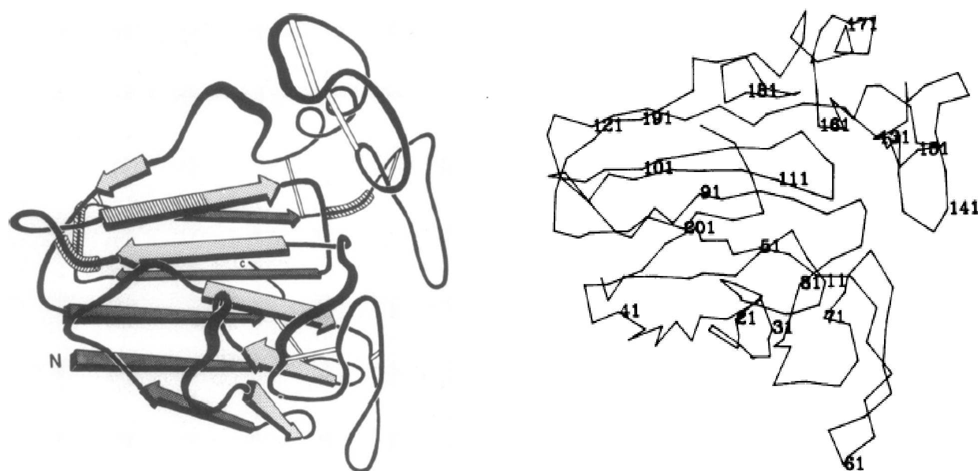

Figure 2.2: (A) Backbone structure of thaumatin I. β strands in the top sheet are shaded light and those in the bottom sheet are darker. Open bars represent disulfide bonds. The viewing direction is along the crystallographic C axis. (B) Stereodrawing of the backbone structure of thaumatin I. (De vos *et al.*, 1985)

Thaumatin produce a persistent sweet sensation over a large part of the tongue in contrast with most sweet compounds which is limited to the front of the tongue. There is an onset of delay before a full sweet taste develops and a slight liquorice aftertaste following it. The sensory effect of thaumatin diminishes slowly, persisting up to half an hour. Thaumatin solution diluted to 10^{-8} M still taste sweet (Witty, 1990). However below this concentration where by sweetness is not detectable, thaumatin acts as a flavour enhancer. It has been used to improve the aroma balance and rounds out the flavours in chewing gum and masking agents in medicines and cigarettes (Gibbs *et al.*, 1996). Its long lasting effect covers strong bitter aftertaste and leaves a pleasant feeling in the mouth. It is therefore useful in masking astringency and off-flavours. Thaumatin can be use to lower the taste threshold of many compounds including savoury flavours at concentration below its sweet threshold. The presence of thaumatin decreases peppermint-taste threshold by 90 % while the beef-extract-taste threshold is reduced by half (Witty, 1990). As a result, thaumatin can be

used as a sweetener, a taste modifier or taste enhancer depending on the type of food and the application.

The usage of thaumatin has been classified as safe by a Joint World Health Organization/Food and Agriculture Organization (WHO/FAO) Expert Committee on Food Additives (JECFA) with no mention of maximum allowable daily intake (ADI). Their finding showed that thaumatin is treated no differently than other proteins with respect to hydrolysis or digestion. Thaumatin showed no mutagenic or teratogenic effects and allergenic effects. The lack of toxicity combined with its ready digestion to normal food components, indicate that thaumatin's only dietary effect is to make an insignificant contribution to the normal protein intake. Thaumatin has received a Generally Recognizes As Safe (GRAS) status in the United States by the Flavor Extract Manufacturers Association (FEMA). In the European Union (EU), thaumatin is listed as food additives E 957. It is permitted as a sweetener, as a flavour enhancer in defined food products (such as ice creams, chocolates preparations and chewing gums) and as in 'flavouring preparation' in all applications under Good Manufacturing Practices. It is approved as a natural food in Japan. Other countries such as Canada, Mexico, Korea, Australia have similar approvals regarding uses as a flavour ingredients. Since it is obtained by a natural process, thaumatin can be labeled as an all-natural ingredient.

2.1.4 Thaumatin extraction

The starting material for thaumatin extraction is the *T. daniellii* fruit which contains up to three seeds. Each seed is coated with a layer of polysaccharide gel and the thaumatin can be found at the soft white brownish aril of the seed. A successful process of thaumatin extraction had been developed by a giant sugar company in Europe, Tate and Lyle as shown in Figure 2.3 (Witty, 1998). The whole fruit is mechanically disrupted exposing the aril to an aqueous solution containing aluminium sulfate and sodium metabisulfate. The sodium metabisulfate acts as an antibrowing

substance and the aluminium salt reduces the swelling of polysaccharide which may absorb thaumatin. The presence of aluminium salt also changes the solution pH to approximately 3.6 and this aid in precipitating contaminated materials. The thaumatin extract can then be bound to cation exchange resin such as sulphopropyl resins either in batch mode or in large columns. The ion exchange is able to remove any browning agents present in the thaumatin extract and thus increasing its purity. The extract is then subjected to ultrafiltration and followed by freeze drying to form an intensely active powder for use in food formulations. Though thaumatin is used at a very low concentration, the presence of aluminium, which is associated with human disease, does not bring an attractive image to it.

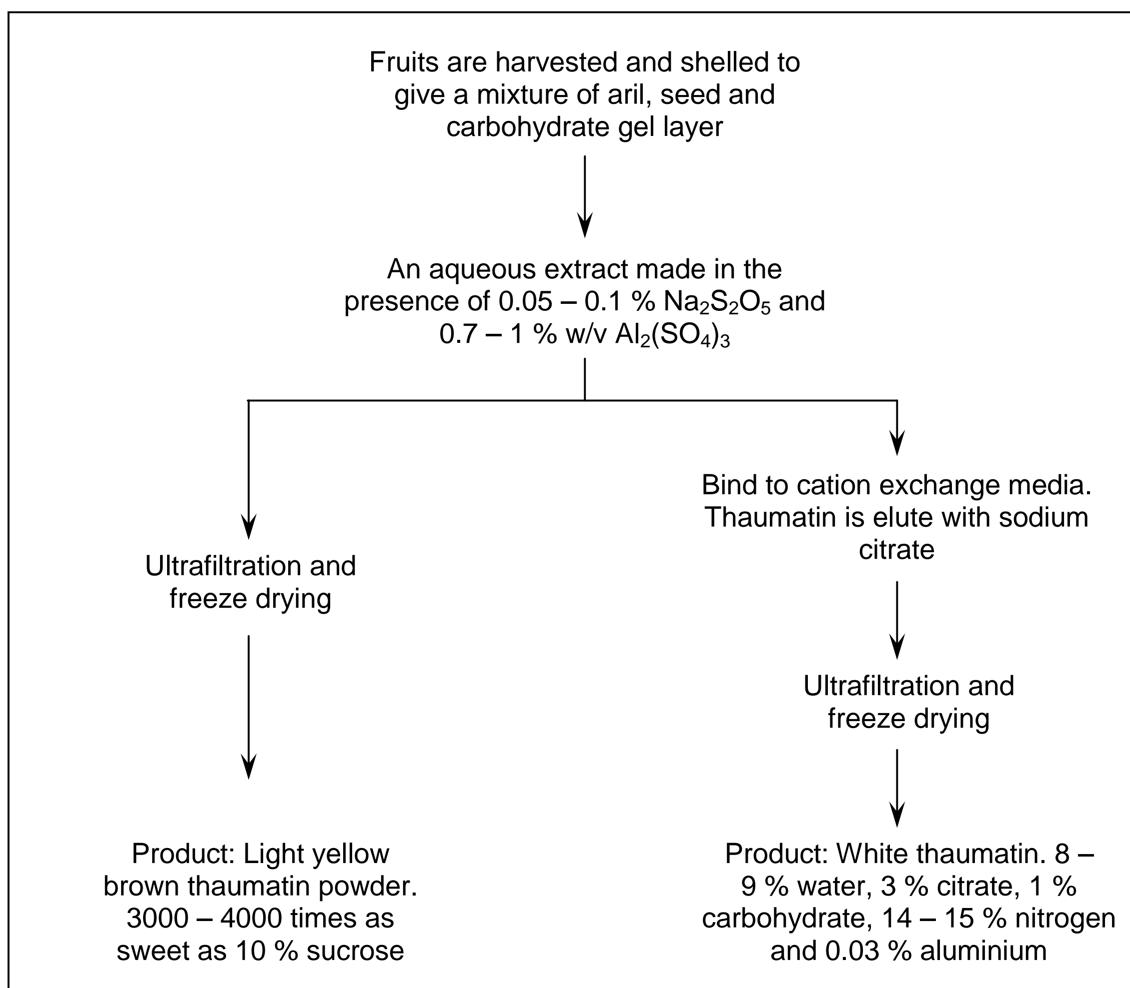

Figure 2.3: Downstream processing of thaumatin (Witty, 1998)

2.1.5 Thaumatin production

Attempts have been made to cultivate *T. daniellii* in the tropic since it is difficult to obtain thaumatin from its natural source. The plants have been grown to maturity in the greenhouses at Reading University, UK, however the flowers abscise without producing any fruits even after artificial pollination (Witty, 1990). Due to this particular drawback, Tate and Lyle PLC which is based in UK, set up plantations in Ghana, Liberia and Malaysia in 1970s to harvest the fruits of *T. danielli* (Jenkins, 2001). The fruits were then frozen and flown to the UK for the rather expensive extraction of the thaumatin protein. Therefore, as an alternative to the production of thaumatin from its natural source, active efforts have been made to produce thaumatin in recombinant host. Production of recombinant thaumatin in several microorganisms and in transgenic plants have been carried out by either using the cloned natural gene for thaumatin II or a synthetic gene that contains codons optimized for expression in the particular host being used. The results of the finding are summarized in Table 2.3. Studies on the production of thaumatin by transgenic plants have also been carried out as well. The expression of thaumatin in plants such as cucumber, tomato, pear and strawberry have been shown to be a success nonetheless, the expression level were still too low for consideration in industrial application (Masuda and Kitabatake, 2006). Detail information with regards to the development in recombinant studies in bacteria, yeast, fungi and plants for sweet protein production can be obtained elsewhere (Masuda and Kitabatake, 2006). At present, the genetically engineered route for the production of thaumatin is far cheaper than harvesting it. However it would only be economically feasible if the recombinant microorganisms could produce thaumatin at 1 g/L (Overbeeke, 1989). So far, none of the attempts at expressing recombinant thaumatin have been capable of reaching these expression levels.

Table 2.3: Most relevant published result on the expression of recombinant thaumatin (Faus, 2000)

Host	Promoter	Secretion	Yield	Sweet phenotype
Escherichia coli	Trp/lac	No	Very low	No
Saccharomyces cerevisiae	PgK	No	Low	No
Kluyveromyces lactis	Gapdh	Yes	Low	No
Bacillus subtilis	α-amy	Yes	1 mg/L	Yes
Streptomyces lividans	β-gal	Yes	0.2 mg/L	-
Penicillium roquefortii	Gla	Yes	1–2 mg/L	Yes
Aspergillus niger var. awamori	Gla	Yes	5–7 mg/L	Yes
Solanum tuberosum	CaMV	No	Low	Yes

Note: Trp/lac, *E. coli* tryptophan and lactose promoters; Pgk, *S. cerevisiae* 3-phosphoglycerate promoter; Gapdh, *K. lactis* glyceraldehydes-3-phosphate-dehydrogenase promoter; α-amy, *B. subtilis* α-amylase promoter; β-gal, *S. lividans* β-galactosidase promoter; Gla, *A. niger* glucoamylase promoter; CaMV, Cauliflower Mosiac Virus promoter for the 35S RNA

2.2 Protein purification and extraction method

Since the recombinant studies have yet to achieve an economically feasible production of thaumain, the direct extraction and purification of thaumatin from *T. daniellii* fruits would be the only source of thaumatin for the time being. Therefore, the development of alternative extraction procedure which counteracts the limitation of the current extraction procedure would be beneficial. The following sections discuss in details on conventional laboratory method as well as aqueous two phase system (ATPS) which could be applied to *T. daniellii* for thaumatin extraction and purification.

2.2.1 Protein precipitation

Precipitation is widely used for product recovery of biomolecules especially protein. Precipitation of protein is a useful method of concentration diluted solutions and is ideal as an initial step in their purification. Precipitation of protein is usually induced by addition of salt, organic solvent or by changing the pH to alter the nature of the solution.

The addition of salt is the most common method of precipitating protein. At low concentrations, the presence of salt stabilizes the various charged groups on a protein molecule, thus attracting protein into the solution and enhancing the solubility of protein (Debye-Huckle theory). This phenomenon is known as salting in. However, as the salt

concentration is increased, a point of maximum protein solubility is usually reached. Further increase in salt concentration leads to decrease number of water molecules available to solubilize the protein. Finally, when there are not sufficient water molecules to interact with the protein molecules, precipitation results (Kirkwood theory). This phenomenon of protein precipitation in the presence of excess salt is known as salting out.

Many types of salts have been employed to precipitate protein by salting out process. The relative effectiveness of the different anions and cations in the salts used for protein precipitation can be obtained from the Hofmeister series (Cacace *et al.*, 1997). Of these salts, ammonium sulfate has been the most widely used salt as it has high solubility, relatively inexpensive, lack toxicity to most enzymes and its stabilizing effect on some enzymes. Reproducible results can be obtained through precipitation provided the protein concentration, temperature and pH are kept constant. In large scale use, however, is limited as it is corrosive except with stainless steel, it forms dense solutions presenting problems to the collection of the precipitate by centrifugation and it may release gaseous ammonia particularly at alkaline pH.

Some protein especially enzymes do not survive ammonium sulfate precipitation. Other salts may be used as substitute but the more favoured alternative is to use organic solvents such as methanol, ethanol, propanol and acetone. Organic solvents precipitate protein by reducing the dielectric constant of the medium and consequently reducing the solubility of the protein by favouring protein-protein rather than protein-solvent interactions. The concentration of organic solvents used is usually kept low although some organic solvents like 2-methyl-2,4-pentanediol (MPD), dimethyl sulfoxide (DMSO) and ethanol can be used in high concentrations. This is because organic solvents have an affinity towards the hydrophobic surfaces of the protein and these results in denaturation of the protein along with precipitation. Organic solvents are not widely used on a large scale because of their cost, their flammability, and the tendency of proteins to undergo rapid denaturation by these solvents if the temperature

is allowed to rise above 0°C. On safety grounds when organic solvents are used, special flameproof laboratory areas are used and temperatures maintained below their flashpoints.

Selective precipitation of protein can also be achieved by changing the pH of the medium. At low pH, proteins have a net positive charge because the amide gains an extra proton. At high pH, they have a net negative charge due to the carboxyl group losing its proton. However, when the pH is equivalent to their isoelectric point (pI), the protein has no net charge. The electrostatic repulsions between the protein molecules are therefore minimum at this condition. This will leads to reduced solubility because the protein is unable to interact with the medium and precipitate out of the solution.

2.2.2 Size exclusion chromatography

Size exclusion chromatography is a chromatographic method in which molecules are separated according to the differences in size or in more technical term, their hydrodynamic volume. It is usually applied to large molecules such as protein. When chromatographic medium is a gel, the technique is more specifically known as gel permeation chromatography or gel filtration chromatography. The gel is a heterogenous phase system in which a continuous liquid phase usually aqueous, is contained within the pores of a continuous solid phase which is the gel matrix. Several types of gels and their chemical stability is listed in Table 2.4. The choice of an appropriate gel depends on the purpose of the experiment and the size of the molecules to be separated. In some cases, it may be important to consider other characteristics of the sample or the molecules to be separated.

In the gel filtration column, the gel particles which are in the form of beads are packed to form a separation bed through which a buffer solution, the eluent, is eluted. Sample molecules which are to be separated are added into the solution as a zone through the top of the bed. The sample zone moves down the bed as eluent is added to the top. The small molecules which diffuse into the gel beads are delayed in their

Table 2.4: Types of gel filtration media and their physiocochemical

Gel type	Chemical and physical properties	Chemical stability
Sepharyl HR	A composite gel prepared by covalently cross-linking allyl dextran with N,N'-methylene bisacrylamide to form a hydrophilic matrix of high mechanical strength. The wet bead diameter is between 25 – 75 mm with an average bead diameter of approximately 50 mm.	pH range 3 – 11. Not affected by detergents, chaotropic salt and dissociating agents. Withstand organic solvents.
Superdex	A composite gel of highly cross-linked porous agarose beads to which dextran has been covalentlly bonded. The high physical and chemical stability are chiefly due to the agarose matrix and the gel filtration properties are principally determined by the dextran chains.	pH range 3 – 12. Withstand strong acid and bases. Not affected by detergents, chaotropic salt and dissociating agents.
Superose	Composed of highly cross-linked porous agarose beads	pH range 3 – 12. Withstand strong acid and bases. Not affected by detergents, chaotropic salt and dissociating agents.
Sephadex	Bead-formed gel is prepared by cross-linking dextran with epichlorohydrin	Stable in aqueous buffer, ionic and non-ionic detergent and dissociating agents
Sepharose	Bead-formed gel prepared from agarose	pH range 4 – 9. Not affected by dissociating agents. Not suitable for chaotropic salts.
Sepharose CL	Gel is prepared from Sepharose by reaction with 2,3-dibromopropanol under strong alkaline conditions. Same porosity as the parent gel but with increase thermal and chemical stability.	pH range 3 – 13. Autoclavable. Withstand organic solvents.

Adapted from Builder (1993)

passage down the column compared with the large molecules which cannot diffuse into the gel matrices and move continously down the column in the flowing eluent. The large molecules thus elute out of the column first followed by the smaller molecules in the order of their sizes.

The resolution depends on the ratio of sample volume to column volume, large sample to column volume ratios gives lower resolution than a smaller one. The recommended sample volumes for obtaining good resolution with different media are given in Table 2.5. The relationship between sample volume, bead size and resolution has been described by Hagel (1985). The actual sample volume for a given separation usually is determined empirically. The resolution can also be tighten by increasing the column length. Increasing the column diameter only increases the capacity of the column but not the resolutions.

Table 2.5: Recommended sample volumes as percentage of the total bed volume for good resolutions

Medium	Recommended sample volume (% of total bed volume)
Sephadex	2 – 5 %
Sepharose	2 – 5 %
Sepharose CL	2 – 5 %
Sepharcyl HR	1 – 2 %
Superdex prep grade	1 – 2 %
Superose prep grade	1 – 2 %
Superdex	0.5 %
Superose	0.5 %

Adapted from Hagel (1985)

2.2.3 Aqueous two phase system (ATPS)

Aqueous two phase system (ATPS) or also known as aqueous biphasic system is a clean alternative for traditional organic-water solvent extraction system. ATPS are formed when two polymers, one polymer and one kosmotropic salt or two salts (one chaotropic and the other kosmotropic salt) are mixed together above their critical concentrations. Two immiscible but predominantly aqueous phases each rich in only one of the two components are formed due to the incompatibility of the components

(Albertsson, 1987). The two phases are mostly composed of water and non volatile components thus eliminating volatile organic compounds which would lead to degradation of biological activities (Albertsson, 1986; Walter *et al.*, 1985).

ATPS have been extensively exploited to process different biological sources for the recovery of biological products. The commonly employed system composes of a polymer-polymer and polymer-salt system. Generally, the former comprises of polyethylene glycol (PEG) and polymer like dextran. In contrast, the later is composed of PEG and phosphate, sulfate or citrate. PEG-dextran system is expensive due to the high cost of dextran. Moreover, dextran, with its large molecular weight, is viscous and thus gives a longer separation time. This system is not considered economically viable for industrial purposes. This therefore had led to trying out of inexpensive substitutes of dextran such as derivatives of starch, cellulose, polyvinyl alcohol, hydroxypropyl starch (HPS) and ethyl hydroxy ethyl cellulose (EHEC). These substitutes are not only inexpensive but can be used at lower concentrations as well. In large scale extraction purposes, PEG-salt system is usually preferred due to the lower viscosity, lower cost of chemical and smaller phase separation time. The polymer-salt system results in higher selectivity in protein partitioning, leading to an enriched product with high yields in the first step of purification.

The implementation of ATPS for the recovery of biological products basically involves the design of extraction stages. In a single stage ATPS process (Figure 2.4), the target product is concentrated at the top polymer rich phase while the bottom phase contains the particles (cells or cells debris) and contaminant (e.g. RNA, carbohydrate, lipids). The potential commercial value of the product, however, will be easily compromised by the high concentration of polymer present in the top phase. Therefore, further processing of the top phase such as with ultrafiltration is required to separate the polymer from the product. In the case of two stage ATPS process, the first extraction eliminates the bottom phase particles and contaminants and generates a top phase enriched with the target product. In the second extraction stage, the product of

interest is partitioned to a bottom salt rich phase which enables the reuse of the polymer rich top phase. Further processing of the salt rich phase by ultrafiltration yields a product concentrate.

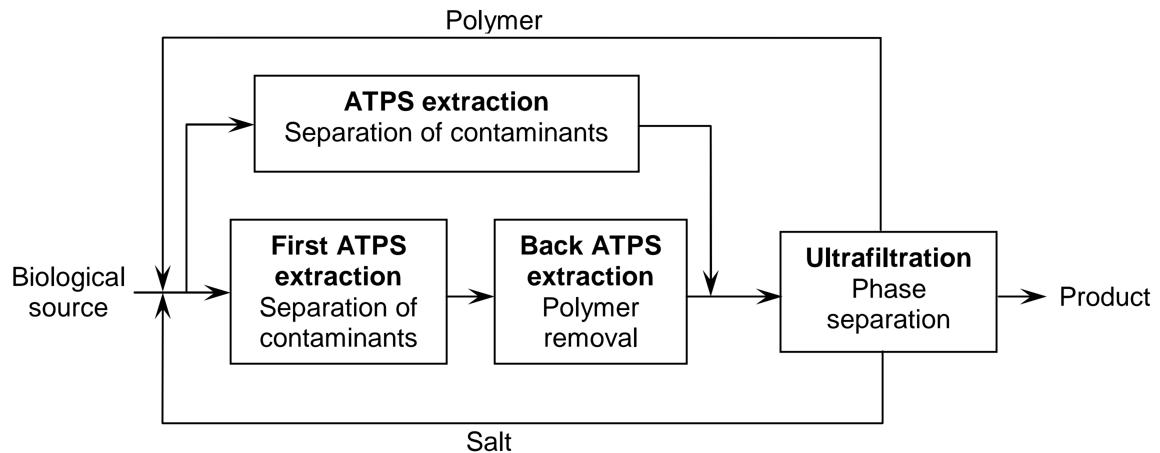

Figure 2.4: Simplified representation of single and two stage ATPS process with ultrafiltration

Table 2.6 is the selection of process applying ATPS for protein recovery. A representative example of the successful extraction process that employs ATPS is the recovery of extra cellular enzyme β-glucoside from *Aspergillus niger* culture filtrate that resulted in a top phase with the protein concentration up to 700 times. The total product recovery was in the range of 85 – 95 % with a concentration factor of 60 – 720 times.

The main advantage of ATPS over conventional technique for biomolecule separation is the high water content of each phase (75 – 95 % w/w) which can be complemented with suitable buffers and salts to provide a suitable environment for biological materials, as well as in an easy scale-up possibilities (Kaul *et al.*, 1995). The polymers used also have a stabilizing effect on most protein. The content of polyols (from the polymer used) present in most aqueous phase media also helps to stabilize the biomolecules by reducing the water content (Johansson, 1985).