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Characterization of a Partially Purified Carom (*Trachyspermum ammi*) Extract and Its Influence on Starch Functionality and Digestibility

A thesis presented in partial fulfilment of the requirements for the degree of Master of Food Technology (MFoodTech) at Massey University (Manawatu Campus), New Zealand

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ABSTRACT

The interactions between starches and the components in spices and herbs have been poorly studied so far. This study investigated the preliminary effects of thirty-six different spices and herbs on pasting properties of rice starch. It largely concentrated on the characterization of a partially purified carom extract (from the dried fruit of the *Trachyspermum ammi* plant) and its influence on the structural, thermal, pasting properties and digestibility of native rice starch. Rheology, differential scanning calorimetry, size exclusion chromatography coupled with a multi-angle laser light scattering, zeta potential, hot-stage optical microscopy, scanning electron microscopy (SEM), and in-vitro starch digestion analysis were carried out to characterise the carom extract and starch-carom system. The results showed that carom, cumin, fennel, mulberry leaf, perilla leaf, neem and coriander seed extracts showed peak and final viscosity-suppressing effect, while mesona, rosemary, green tea, thyme, and clove extracts showed peak viscosity-enhancing effect on rice starch during starch pasting. The water-soluble fraction of carom had the highest degree of viscosity-suppressing effect as compared to other spices and herbs. With increasing concentration of carom, the peak and final viscosities of rice starch decreased; the onset, peak, and end temperatures of rice starch increased; and granular swelling of potato starch was restricted and delayed. The viscosity-suppressing effect was not caused by pH or small molecular carom compounds such as mineral salts and phytochemicals. A protein polymer in carom extract with an M_w of ~2.08 $\pm$ 0.10 x 10^5 Da and isoelectric point of ~3.5 was found responsible for the suppression effect. The protein fraction completely denatured at ~83^{o}C. Micrographs of SEM showed that carom protein appeared as raisin-like clusters. The ability of carom protein to suppress the peak viscosity of starch was also observed in potato, tapioca, glutinous rice, waxy maize, waxy rice, rice, sweet potato, maize, wheat, and pea starches, suggesting that the effect was independent of the source and ratio of amylose to amylopectin. It was proposed that the protein molecules could be interacting with the starch granular surface and/or starch molecules. *In-vitro* starch digestion study showed that dialysed carom extract with rice starch caused an unusual increment in glucose release. The lower viscosity of the starch-carom gels and/or a carom enzyme stimulatory effect were proposed to be responsible for increasing the rapid breakdown of starch.

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CHAPTER 1: GENERAL INTRODUCTION

Spices and herbs have been used for centuries as flavourings, colourings, medicines, preservatives and perfumes throughout the world (Uhl, 2000a). The leaf, root, bark, fruit, seed or flower of a plant used as ingredients for the purpose of cooking are commonly termed as spices and herbs (Opara & Chohan, 2014). Besides the highly valued sociocultural and culinary significance, spices and herbs have several modern pharmaceutical and nutraceutical applications (Patch, 2006; Tapsell et al., 2006). This is due to the various bioactive macromolecules, micronutrients, and phytochemicals (e.g. terpenoids, phenolic compounds, glucosinolates, betalains, chlorophylls, and other organic acids) found in spices and herbs that have biological effect on human health (Pradeep, Geervani, & Eggum, 1993; Uhl, 2000b).

Spices and herbs are commonly used in culinary applications to add colours, aromas, or flavours to starch-based foods. Carom, cinnamon, dill and ginger are usually incorporated into bread doughs, whereas bay leaf, fenugreek, turmeric and tamarind are added to rice before cooking (Uhl, 2000b). The macromolecules or phytochemicals in these spices and herbs may interact with starch or other components in foods to influence digestion and absorption of nutrients (Adefegha & Oboh, 2012; F. Barros, Awika, & Rooney, 2012; Srinivasan, 2005b; Fan Zhu, 2015). Up to the present time, there are insufficient studies in this area.

Starch is an important ingredient in many foods and a staple in the human diet. When subjected to thermal treatment in the presence of water, starch undergoes gelatinization, and retrogradation occurs upon cooling (Q. Liu, 2005). Both gelatinization and retrogradation are influenced not only by the starch composition and structure but also by the environmental conditions such as water activity, pH, temperature, and presence of additives (e.g. macromolecules, sugars, ions, and phytochemicals). Completely gelatinised starches may induce high peaks in the blood glucose levels, which suggest that the food may have a high glycemic index (GI) value or glycemic impact (Parada & Aguilera, 2009; Tosh & Chu, 2015; Widanagamage, Ekanayake, & Welihinda, 2013). Diets that mostly comprises of rapidly digested starches can lead to

sustained elevation of blood glucose levels and increase the risk of various diseases such as diabetes, obesity, cardiovascular diseases and certain cancers (Brand-Miller, Dickinson, Barclay, & Celermajer, 2007; Brand-Miller, McMillan-Price, Steinbeck, & Caterson, 2009; Buyken et al., 2014; Riccardi & Rivellese, 2000).

Recently, the incorporation of certain spices and herbs (e.g. green tea) with starches have been reported to possibly restrict gelatinisation or have an enzyme inhibitory effect to prevent the rapid breakdown of starch (R. Goh et al., 2015; Quek & Henry, 2015). In contrast, certain spices and herbs (e.g. cumin) may aid in the digestion process of foods by stimulating bile acid production, enhancing pancreatic and small intestinal enzymes activities, and reducing the food transit time (Platel, Rao, Saraswathi, & Srinivasan, 2002; Platel & Srinivasan, 2000a, 2004; Srinivasan, 2005a, 2005b). These digestion-stimulating activities may result in rapid breakdown of food macromolecules. However, the mechanism of stimulation is still poorly understood.

To date, interactions between starches and the active components in spices and herbs are poorly studied. The effect of these components on the rheological, thermal, chemical, and structural properties and digestibility of starches are currently unknown. Knowledge of the interactions between starch and components in spices / herbs will provide new avenues for the food and nutraceutical industries to develop and design products with certain functional and nutritional properties. This development could also reverse the need for chemical modification of starch and enable “clean labels” in food products (Bemiller, 2011). This study investigated the preliminary effects of different spices and herbs on starch pasting / rheological properties. It specifically focused on an Indo-Persian spice called carom (dried fruit of the *Trachyspermum ammi* plant), which is traditionally valued for its digestion simulative and carminative properties. Therefore, this study majorly concentrated on the characterization of a partially purified carom extract and its influence on the structural, thermal, and pasting properties and digestibility of native rice starch.

The objectives of the study were to:

- Screen the preliminary effects of thirty-six different spice and herbal extracts on the pasting properties of native rice starch.
- Determine the effect of partially purified carom extract on the pasting properties of rice starch.
- Determine the components (e.g. polysaccharides, lipids, proteins, sugars, acids, bases, minerals, phytochemicals, or enzymes) in carom extract that can influence starch pasting properties.
- Characterise the physicochemical properties of partially purified carom extract.
- Understand the rheological, thermal, chemical, and structural properties of starch-carom systems.
- Study the effect of partially purified carom extract on the digestibility of rice starch using an *in-vitro* gastrointestinal digestion model.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

The aim of this section was to review the physico-chemical properties of starch and spices / herbs, and their possible interactions. To date, no literature is available to explicate the direct interaction between these two materials. A brief overview of the different spices and herbs and a comprehensive review of carom's composition and biological properties are presented in this chapter. This current published knowledge provides information to understand how the different fractions in spices / herbs (macromolecules, sugars, acids, bases, mineral salts, enzymes, or phytochemicals) may influence pasting properties and digestibility of starch.

2.2 Spices and Herbs

Spices and herbs are commonly used for culinary, sociocultural, and ethno-medicinal purposes throughout the world (Uhl, 2000a). Spices are generally defined as "a non-leave component of plant that imparts flavour to food" and examples would include cinnamon and carom. Herbs are defined as "plant whose leaves are used to impart flavour or in making medicine" and examples would include rosemary and dill (Uhl, 2000a). The phytochemicals in these spices and herbs impart flavours, colours, and aromas to food. Spices and herbs also possess various therapeutic effects that allows its usage in traditional and modern medicines. For example, the drug aspirin (acetylsalicylic acid) was synthesised based on a compound found in white willow bark (*Salix alba*) used in herbal medicine. To date, there are insufficient randomized-controlled human trials to establish efficacy of all herbal medicine in the treatment of diseases. However, preliminary animal and *in-vitro* studies have shown anti-oxidant, anti-carcinogenic, and anti-inflammatory properties in many spices and herbs (Reddy, Reddy, & Jamil, 2015; Tapsell et al., 2006). The effects of spices and herbs on the physical properties of foods are still not well studied. This could be due to the complexity of macromolecules and phytochemicals present in them. Table 1 shows the major bioactive chemical compositions and ethno-medicinal properties of thirty-six spices and herbs that were screened in this study (*refer to Figure A1 in Appendices for the images of the plant*).

Table 1: *Major chemical components and health properties of 36 different spices and herbs.*

Common Name	Family	Binomial name	Major Chemical Components	Ethno-Medicinal Usage
Carom	Apiaceae	*Trachyspermum ammi*	Thymol, γ-terpinene, ρ-cymene, β-pinene, myrcene, carvacrol	Flatulence, dyspepsia, diarrhoea, cholera, stomach disorders
Coriander Leaf	Apiaceae	*Coriandrum sativum*	2-decenoic acid, E-11-tetradecenoic acid, capric acid, undecyl alcohol	Diarrhoea, high blood cholesterol, ulcers, anaemia
Coriander Seed	Apiaceae	*Coriandrum sativum*	D-linalool, α-pinenes, b-terpinene, camphor, limonene, ρ-cymene	Diarrhoea, high blood cholesterol, ulcers, anaemia
Cumin	Apiaceae	*Cuminum cyminum*	Cuminic aldehyde, β-pinene, terpinene, ρ-cymene	Flatulence, indigestion, diarrhoea, nausea, morning sickness
Dill	Apiaceae	*Anethum graveolens*	Carvone, limonene, a-phellandrene, p-cymene	Gastro-intestinal disorders and spasm
Fennel	Apiaceae	*Foeniculum vulgare*	Trans-anethole, fenchone, limonene, a-phellandrene, α-pinene, a-thujene	Flatulence, indigestion, diarrhoea, nausea, morning sickness
Parsley	Apiaceae	*Petroselinum crispum*	Myristicin, apiole, α-pinene, β-phellandrene, myrcene, limonene	Stomach disorders, menstrual problems, arthritis and colic
Codonopsis Root	Campanulaceae	*Codonopsis pilosula*	Phenols, baicalein glucosinolates, saponins, trace alkaloids	Fatigue, diarrhoea, vomiting, cough
Chinese Yam	Dioscoreaceae	*Dioscorea polystachya*	Diosgenin, mucilage, choline, starch, sugar, protein, free amino acids	Spleen disorders, stomach disorders, kidney disorder, cough
Red Bush Tea	Fabaceae	*Aspalathus linearis*	Flavanols, flavones, flavanones, dihydrochalcones, aspalathin	Restlessness, vomiting, and stomach cramp
Reishi Mushroom	Ganodermataceae	*Ganoderma lucidum*	Terpenoids, steroids, phenols, nucleotides, glycoproteins	Diabetes, weak immunity, liver disorder and bacterial infections
Holy Basil	Lamiaceae	*Ocimum tenuiflorum*	Ugenol, carvacrol, nerol, eugenol methylether	Bronchitis, respiratory disorders, stress, anxiety
Mesona (Grass Jelly)	Lamiaceae	*Platostoma palustre*	N-hexadecanoic acid, linoleic acid, linolenic acid	Hypertension, diabetes and liver disease
Mint	Lamiaceae	*Mentha spicata*	Carvone, dihydrocuminyl acetate, dihydrocuminyl valerate	Colic, vomiting, hysteria, cough, stomach disorders
Oregano	Lamiaceae	*Origanum vulgare*	Terpinen-4-ol, α-terpinene, a-terpineol, sabinene, linalool	Cough, spasm, vomiting, indigestion, parasitic infections
Perilla Leaf	Lamiaceae	*Perilla frutescens*	Perillaldehyde, elsholtziaketone, perilla ketone	Nausea, vomiting, motion sickness, restless foetus
Rosemary	Lamiaceae	*Rosmarinus officinalis*	1,8- cineol, borneol, camphor, bornyl acetate, α-pinene	Pain, stress, anxiety, tension, scalp disorders
Sage	Lamiaceae	*Salvia officinalis*	Thujone, 1,8-cineol, borneol, camphor, camphene, a-humulene	Bacterial infection, spasm, cough

Thyme	Lamiaceae	*Thymus vulgaris*	Thymol, carvacrol, 1,8-cineole, p-cymene, linalool, borneol, α-pinene	Bacterial infection, spasm, cough, parasitic infection
Cinnamon	Lauraceae	*Cinnamomum cassia*	Cinnamic aldehyde, eugenol, linalool, cinnamyl acetate, safrol, 1,8-cineole	Indigestion, flatulence, coughing, cramps, viral infections, colic
Neem	Meliaceae	*Azadirachta indica*	Quercetin, nimbosterol, limonoids, tannins, azadirachtin, azadiradione	Diabetes, bacterial infection, viral infection, fever
Mulberry Leaf	Moraceae	*Morus alba*	Steroids and triterpenoids, flavonoids and its glycosides, coumarin	Coughs, respiratory disorder, liver disorder
Nutmeg	Myristicaceae	*Myristica fragrans*	Sabinene, α-pinene, b- pinene, myrcene, 1,8-cineole, myristicin	Nausea, vomiting, flatulence
Clove	Myrtaceae	*Syzygium aromaticum*	Eugenol, eugenyl acetate, b-caryophyllene, a- and b-humulene	Pain, vomiting, bacterial infection, tooth ache
Indian Gooseberry	Phyllanthaceae	*Phyllanthus emblica*	Vitamin C, gallic and ellagic acids	Immunity, rejuvenate skin, premature aging
Black Pepper	Piperaceae	*Piper nigrum*	Sabinene, α-pinene, β-pinene, limonene, 1,8-cineol	Bacterial infection, constipation, vertigo, sore throat, poor appetite
Brahmi	Plantaginaceae	*Bacopa monnieri*	Brahmine, herpestine, saponins, monnierin, hersaponin, bacosides	Learning, academic performance, improves mental ability
Loguat Leaf	Rosaceae	*Eriobotrya japonica*	Erolidol, farnesol, amygdalin, tartaric acid	Urinary disorders, wind, stomach disorders, tension
Coffee	Rubiaceae	*Coffea arabica*	Caffeine, Trigonelline, Chlorogenic acids, Quinic acid	Stimulant
Star Anise	Schisandraceae	*Illicium verum*	Anethole, α-pinene, phellandrene, p-cymene, 1,4-cineol, limonene	Colic, stomach pains, indigestion, sore throats, coughs
Black Tea	Theaceae	*Camellia sinensis*	Catechins, theaflavins, thearubigens, flavonols, phenolic acids	Heatiness, thirst, phlegm, indigestion, bowel disorder, headache, dizziness, sleepiness, urinary disorders
Green Tea	Theaceae	*Camellia sinensis*	Catechins, flavonols, theogallin, depsides, gallic acid, quinic acid	
Oolong Tea	Theaceae	*Camellia sinensis*	Monomeric catechins, theaflavins and thearubigins	
Cardamom	Zingiberaceae	*Elettaria cardamomum*	1,8-cineole, a-terpineol acetate, linayl acetate, sabinene, limonene, linalool	Heartburn, bloating, dyspepsia, cough, headache
Ginger	Zingiberaceae	*Zingiber officinale*	Zingiberene, curcumene, α-pinene, sabinene, limonene, borneol, linalool	Nausea, morning sickness and motion sickness
Turmeric	Zingiberaceae	*Curcuma longa*	Zingiberene, curcumene, α- and β-turmerone, curcuminoids	Liver damage, respiratory disorders, ulcers, joints aches

Source: (Kaefer & Milner, 2008; Opara & Chohan, 2014; Patch, 2006; Platel & Srinivasan, 2004; Pradeep et al., 1993; Uhl, 2000b)

2.3 Carom (*Trachyspermum ammi)*

2.3.1 Classification and characteristics

Trachyspermum ammi (L.) Sprague is an annual herbaceous plant belonging to the Apiaceae (Parsley) family. The Apiaceae family includes plants such as coriander, dill, fennel, and cumin (Table 1). *Trachyspermum ammi* is indigenous to Egypt and is widely cultivated in the Middle East and South Asia (Zarshenas, Moein, Samani, & Petramfar, 2014). Carom, which is the common name of the dry fruit pots (schizocarp) of the *Trachyspermum ammi* plant, is used as a spice for culinary, perfumery, and medicinal purposes (G. Singh, Maurya, Catalan, & De Lampasona, 2004; Uhl, 2000b). Carom is also closely related to wild celery plant (*Trachyspermum roxburghianum*), which is also used as a spice in South and South-East Asia. Pictures of *Trachyspermum ammi* plant and carom spice are shown in Figures 1a and 1b respectively.

			
Figure 1a: *The Trachyspermum ammi plant and flower.*	***Figure 1b:*** *Carom, the* schizocarp (dry fruit) *of the Trachyspermum ammi plant, used as a spice.*	***Figure 1c:*** *Omum water, a home remedy concoction made solely from carom aqueous extract.*	***Figure 1d:*** *An Indian traditional medicine, Gasex Syrup, containing carom.*

Source: (Asif, Sultana, & Akhtar, 2014; The Himalaya Drug Company, 2014).

Carom is known by different names such as Bishop's Weed (English), Yavani (Sanskrit), Ajowan (Spanish, German, French), Taleb-El-Koubs (Arabic), Ajwain / Carom (Hindi), Xi Ye Cao Guo Qin (Chinese), Ayowan (Korean), and Omam (Tamil) (Uhl, 2000b). It has a light brown colour with a large curved and ridged oval appearance that is similar to celery seeds (Figure 1b). Carom has a characteristic piney, phenol-like, bitter and spicy note (Uhl, 2000b).

2.3.2 Traditional medicine and culinary applications

In Indian Traditional Medicine (Ayurveda) and Tamil Traditional Medicine (Siddha Maruttuvam), carom is valued for its antispasmodic (suppresses gastrointestinal spasms), digestion simulative, carminative (suppresses formation of gas in the gastrointestinal tract) and anti-microbial properties. It is administered for individuals with flatulence, atonic dyspepsia (indigestion), diarrhoea, poor appetite, respiratory problems, abdominal tumours, and piles (Bairwa, Sodha, & Rajawat, 2012).

In Indian communities in South India and South-East Asia, the aqueous extract of carom, known as “Omum Water” (Figure 1c), is commercially available for relieving indigestion in children and adults. Standardised herbal formulations (Figure 1d) containing carom are also widely used throughout India for gastrointestinal disorders. In ethno-veterinary medicine, whole carom are commonly used to treat camels suffering from gastrointestinal disorders in India (Sharma & Manhas, 2015).

In Islamic Medicine (Unani) practised in South Asia, carom is used for amoebiasis (parasite infection) and gastrointestinal disorders. In Traditional Iranian Medicine (Irani-Tebb), carom is used for nausea, vomiting, acid reflux, abdominal cramps and loss of appetite (Zarshenas et al., 2014). Carom is occasionally used in Ancient Greek Medicine and Traditional Chinese Medicine (Zhōngyī) for similar purposes. However, it crucial to note that the number of randomized controlled human trials and systemic reviews to prove the efficacy of carom’s therapeutic effects are limited.

In culinary application, carom is an important ingredient in South Asian, African, and Middle Eastern cuisines (Uhl, 2000b). In India, it is added to root vegetables and legumes for flavour, to ease digestion and prevent flatulence. The spice is also incorporated into commonly consumed pan-fried breads (Figure 2a), deep-fried snacks (Figure 2b), and pastries (Figure 2c). In Ethiopia and Eritrea, carom is an integral part of Berberé spice mix, which is added to stews (Figure 2d).

			
Figure 2a: *Mirch Ajwain Paratha, an Indian pan-fried bread made from wheat flour and carom. A common staple in North India.*	***Figure 2b:*** *Omumpodi Muruku, an Indian deep-fried snack made from chickpea flour, rice flour, and carom.*	***Figure 2c:*** *Ajwain Samosa, an Indian deep-fried stuffed pastry made from flour and carom. A common snack in India.*	***Figure 2d:*** *Ethiopian Berberé stew, made from carom and other spices. Berberé is a key ingredient in Ethiopian cuisines.*

Besides traditional medicine and culinary application, Korean scientists have evaluated the unique possibilities of using carom extract for agricultural and pest control purposes as well. Carom extract has shown to have strong fumigant and insecticidal activity against German cockroaches, Japanese termites, and Asian tiger Aedes mosquito (S.-M. Seo et al., 2009; Seo et al., 2015; S. M. Seo et al., 2009; Seo, Park, & Park, 2012). In addition, carom has been reported to have potent inhibitory activity against barnyard grass plant (major agricultural nuisance to farmers) and three Aspergillus species responsible for disease in animals and plants (Chung, Khanh, Lee, & Ahmad, 2007; E. Kim et al., 2015).

2.3.3 Chemical constituent

Table 2 shows the compositions of whole carom. It was reported to be particularly high in fat, protein, and insoluble fibre content (Pradeep et al., 1993). The fat-soluble phytochemicals in the essential oil of carom have been extensively studied. However, the protein, soluble fibre and other water-soluble fractions are poorly characterised. Like most other schizocarps (dry fruits), the husk and other cellulose-like materials contribute to the high amount of insoluble fibre content.

Table 2: *Composition of whole carom*

Composition	Unit	Amount
Proximate Composition		
Moisture	(g/100 g)	7.20
Ash		7.81
Protein		16.50
Fat		30.99
Other Components*		37.50
Total:		**100.00**
Mineral Composition		
Calcium	(mg/100 g)	1252.80
Phosphorus		380.50
Iron		53.82
Manganese		4.73
Magnesium		259.80
Zinc		6.31
Saccharide Composition		
Starch	(g/100 g)	0.90
Total Sugars		3.53
-Glucose		0.30
-Fructose		0.19
-Fructans		0.46
-Sucrose		2.58
Total Dietary Fibre		48.90
-Insoluble		42.96
-Soluble		5.91

Source: (Pradeep et al., 1993)

** Other unknown components may possibly refer to carbohydrates and/or phytochemicals*

Table 3 shows the quantitative analysis of the general phytochemical content in carom using thin layer chromatography. Carom contains particularly high amounts of water-soluble phytochemicals such as tannins and flavonoids (Kaur & Arora, 2009). Saponins (amphipathic glycosides) and alkaloids (low water-solubility) are also present in carom (Kaur & Arora, 2009). There has been 36.29% of phytochemicals quantified in carom (Table 3), which corresponds closely to the 37.50% of unknown components stated in Table 2.

Table 3: *Quantitative (%) phytochemical evaluation of carom*

Phytochemical Composition of Carom		
Alkaloids	%	4.23
Flavonoids		8.58
Tannins		22.77
Saponins		0.71
Total		**36.29**

Source: (Kaur & Arora, 2009)

The amount of essential oil extracted from whole carom can vary from 2 to 5% (w/w). Table 4 shows the Gas Chromatography-Mass Spectrometry chemical analysis of carom's essential oil reported in different studies. Table 4 shows that thymol, γ-terpinene, ρ-cymene, β-pinene, myrcene, and carvacrol are major constituents of carom essential oil. The differences in the chemical composition published in different studies could be due to maturity, season of harvest, and geographical location of the plants (Gandomi, Abbaszadeh, JebelliJavan, & Sharifzadeh, 2014).

Table 4: *Major chemical composition (%) of carom's essential oil*

Phytochemical Name	[1]	[2]	[3]	[4]	[5]
	%				
Thymol	41.77	54.50	45.60	24.11	63.42
γ-terpinene	27.77	22.96	20.00	28.66	16.89
ρ-cymene	24.4	19.38	11.03	33.06	19.01
β-Pinene	1.26	0.78	6.04	6.74	
Myrcene	0.48	0.48	1.01	1.18	
Carvacrol	0.55	0.46	2.87	0.50	
β-Phyllanderene			2.05	0.65	
(R)-(+)-α-pinene	0.87	0.10	1.85	0.65	0.06
α-Thujene		0.30	2.65	0.54	0.07

Source: [1] (S.-M. Seo et al., 2009); [2] (Mohagheghzadeh, Faridi, & Ghasemi, 2007); [3] (Kazemi, 2014); [4] (Chalchat, ÖZcan, & Figueredo, 2011); [5] (Gandomi et al., 2014)

Table 5 shows that all the major phytochemicals found in carom essential oil belong to the monoterpenoid of the prenol lipid category. These monoterpenoids are characteristically less soluble in water and are usually found in the lipid extract of carom.

Table 5: *Classification and solubility of main phytochemicals in carom essential oil*

Name	Phytochemical Classification	Solubility in water (25°C)
Thymol	Cyclic Monoterpenes (Monoterpenoid)	980.0 mg/L
γ-terpinene		N/A
ρ-cymene		23.4 mg/L
β-pinene		4.9 mg/L
Carvacrol		N/A
Myrcene	Linear Monoterpenes (Monoterpenoid)	5.6 mg/L

Source: (National Center for Biotechnology Information, 2016)

2.3.4 Aqueous extract of carom

The aqueous extract of carom is the focus of interest throughout this study. Therefore, a thorough review on the aqueous extract is presented in this sub-section, though little information is known about it. To date, a bioactive protein and certain phytochemicals (such as tannins, flavonoids, monoterpenoids, glucosides, nucleosides, and glucides) have been reported in the aqueous extract (Ishikawa, Sega, & Kitajima, 2001; T. Kaur, R. K. Bijarnia, S. K. Singla, & C. Tandon, 2009).

An *in-vitro* study showed that the aqueous extract of carom had an anthelmintic (anti-parasitic) effect comparable to albendazole (broad-spectrum anti-parasitic drug) (Apte, Khot, Biradar, & Patil, 2014). However, the active compound in the aqueous extract was not identified in the study but was suggested to be an attribute of thymol (fat-soluble monoterpenoid). In another study, phenolic compounds and flavonoids in the aqueous extract inhibited seven bacterial species known to cause diarrhoea (J. C. Rao, Gadkari, Chouta, & Sagar, 2014).

In an *in-vivo* animal study, the extract was shown to prevent ethanol-induced gastric mucosal damage and gastric ulcers (Tajik, Kheirandish, Amanollahi, & Shahabi, 2015). The anti-inflammatory properties of the extract decreased the inflammatory cell infiltration and provided a gastro-protective healing effect in rats (Tajik et al., 2015). Carom aqueous extract has also shown antiepileptic (anti-seizure) and anxiolytic (anti-anxiety) effects in animal studies (Rezvani et al., 2011). It was suggested that the psycho-active properties could be mediated by phytochemicals or amino acids (e.g. L-lysine) (Rezvani et al., 2011).

A bioactive protein isolated from carom aqueous extract was first reported by Kaur et al. (2009). The protein showed significant anti-lithiatic properties (prevents the formation of kidney stones) in an *in-vivo* model and calcium binding properties in an *in-vitro* model (Aggarwal, Singla, & Tandon, 2014; Tanzeer Kaur, Rakesh K. Bijarnia, Surinder K. Singla, & Chanderdeep Tandon, 2009). The anti-calcifying protein had a molecular weight of 107 kDa and isoelectric point of 6.2 and was reported to contain high level of acidic amino acids (aspartic acid and glutamic acid) (T. Kaur et al., 2009). No further studies have been reported on the protein characteristics or functionality.

2.4 General Properties of Starches

2.4.1 Starch molecules

Starch found in many plants provides the main source of energy in the human diet (Colonna & Buleon, 2009). Starch has been isolated as an ingredient used in various food applications for their thickening, gelling, stabilising, clarifying, and fat-replacing properties (Andréa Curiacos Bertolini, 2009). The major sources of starch include cereal grains (maize, rice, wheat, barley, oat, and sorghum), root crops (sweet potatoes, cassava, arrowroots, and yam), tubers (potatoes), stems (sago palm), and legumes (peas and beans) (M. A. Rao, Tattiyakul, & Liao, 2012).

Starch is essentially a condensation polymer of glucose molecules connected by acetal linkages (Imam et al., 2012). It primarily consists of two components called amylose and amylopectin. Amylose is mostly a linear polymer that consists of several thousand (1→4)-α-D-linked D-glucose units with some molecules that are slightly branched by (1→6)-α-linkages (Peris-Tortajada, 2015; N. Singh, Singh, Kaur, Sodhi, & Gill, 2003). Amylopectin is the highly branched polymer that consists of short segments of (1→4)-α-D-linked D-glucan units connected by (1→6)-α-D-glycosidic linkages (Peris-Tortajada, 2015). The molecular mass for amylose and amylopectin ranges from 2×10^5 to 2×10^6 g/mol and from 1×10^6 to 5×10^8 g/mol respectively (Peris-Tortajada, 2015). The schematic representation of the structure of a starch granule and occurrence of amylose and amylopectin is shown in Figure 3.

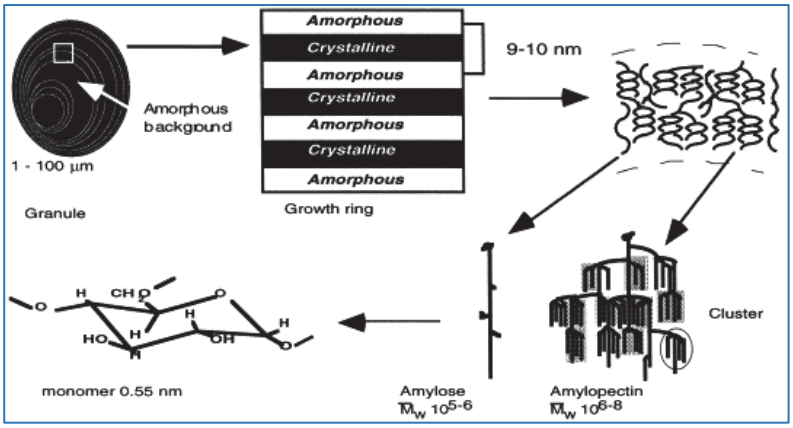


Figure 3: *Schematic representation of the different structural levels of the starch granule and the occurrence of amylose and amylopectin. Source: (N. Singh et al., 2003)*

Figure 3 shows that amylose and amylopectin polymers are in the form of a semi-crystalline macromolecular structure (Mitolo, 2005). The amylose and amylopectin are deposited in successive layers around the central hilum. Crystalline micelles hold the granule together that allows it to remain intact (Mitolo, 2005).

2.4.2 Minor components

Besides amylose and amylopectin, starch granules also contain small quantities of proteins, lipids, pentosans, enzymes, amino acids, nucleic acid, and minerals (Baldwin, 2001). Proteins and lipids are the most abundant among the minor components. Cereal starches typically contain ~0.25% protein and up to ~1.0% lipids, while root or tuber starch contains ~0.05% protein and 0.05 – 0.1% of lipids (Baldwin, 2001). The proteins can be further classified into: (i) storage proteins (e.g. gliadin and glutenin) and (ii) starch granule-associated proteins (e.g. puroindoline and starch synthase protein), while the lipids are classified into: (i) lysophospholipids and (ii) starch granule-associated lipids (Baldwin, 2001).

These proteins and lipids appears to significantly influence the overall properties of the starch granules (Baldwin, 2001). For example, high amount of lipid content in cereal starches allows hydrophobic interactions between amylose and lysophospholipids that form amylose-lysophosphatidylcholine complexes (W. Wang, Yang, & Cui, 2015). These amylose-lipid complexes reduce the amount of water that can access the starch granules and as a result restrict the granular swelling during heating (W. Wang et al., 2015).

Puroindoline is one of the ten major starch granule-associated proteins found on the surfaces of cereal starch granules. Puroindoline has been reported to increase the bread loaf volume and stabilize bread crumb texture through a puroindoline–gluten interaction (Igrejas et al., 2001). The addition of soy protein to wheat-based dough has also shown a puroindoline-soy protein interaction through hydrophobic interactions in the presence of heat (Ryan & Brewer, 2005b, 2006). These studies suggest that granule-associated proteins serve as sites of attachment for existing gluten and other added protein molecules in the wheat-based dough systems (Ryan & Brewer, 2006). A further study has also shown that the removal of granule-

associated puroindoline protein decreased the binding of added soy proteins, which suggest that granule-associated proteins play an important role in mediating the binding of exogenous proteins (Ryan & Brewer, 2007). Therefore, it is known that amylose, starch granule-associated lipids, and starch granule-associated proteins have hydrophobic sites that allow hydrophobic interactions between components.

2.4.3 Types of starches

Table 6 shows the proximate proportions of amylose and amylopectin found in different starches. However, the value reflected in the table may vary depending on the maturity of the plant, season of harvest, cultivar, and geographical location.

Table 6: *Characteristics and properties of common starches*

Starch Type	Amylose Content (%)	Amylopectin Content (%)	Swelling Power (%)*	Gelatinisation Range (°C)
Maize (Waxy)	1	99	64	63-74
Rice (Waxy)	1	99	N/A	64-79
Glutinous Rice	1-4	96-99	N/A	71-87
Rice	17	83	19	61-80
Tapioca	17	83	71	52-64
Sweet Potato	21-24	76-79	N/A	55-69
Potato	21-22	78-79	1000	56-69
Wheat	25-28	72-75	21	53-72
Maize (Native)	26-28	72-74	24	62-80
Pea	21–34	66-79	17	55-80
Maize (High)	50-85	15-50	6	85-87

Sources: (Collado & Corke, 2003; S. Damodaran, K.L. Parkin, & O.R. Fennema, 2008; Henry & Alistair, 2006).
* The classical swelling power (SP) is defined as the wet weight of the sedimented gel divided by its dry weight (Leach et al., 1959).

Swelling during gelatinisation is a property of amylopectin that generally results in a high peak viscosity in a pasting curve during heating (Fredriksson, Silverio, Andersson, Eliasson, & Aman, 1998; Tester & Morrison, 1990). Due to the branched nature, amylopectin is less prone to gelation and retrogradation that results in a lower final viscosity during the cooling process (A. Brown, 2008; Collado & Corke, 2003; S. Damodaran et al., 2008). Generally, waxy and tuber starches with inherently high amylopectin contents will have higher peak viscosity and lower final viscosity than high amylose starches.

Conversely, amylose actively inhibits swelling during heating due to the strong hydrogen bonds holding the amylose molecules together (Fredriksson et al., 1998; Tester & Morrison, 1990). However, during gelation and retrogradation, the amylose molecules form bonds rapidly to create a three-dimensional network and increase the final viscosity of the system (A. Brown, 2008; Collado & Corke, 2003; S. Damodaran et al., 2008). Generally, cereal and pea starches with inherently high amylose content have a lower peak viscosity and a higher final viscosity than high amylopectin starches.

2.4.4 Starch pasting properties

2.4.4.1 Starch pasting curve

A standard starch pasting curve consisting of a heating and cooling cycle is schematically represented in Figure 4. The important characteristics of a starch pasting curve include pasting temperature point (B), peak viscosity (D), holding strength point (E), and final viscosity (F). The term “starch pasting properties” is used throughout this study to denote a typical starch pasting curve that comprises of gelatinisation, pasting, breakdown, and gelation.

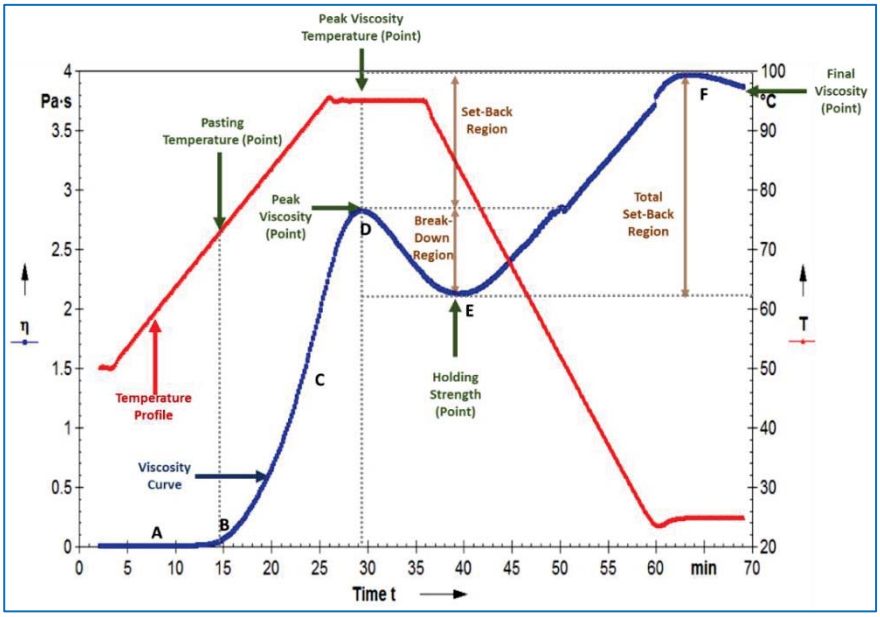


Figure 4: *A typical starch pasting curve of 10% (w/w) native rice starch in Milli-Q water*

2.4.4.2 Hydration

Native starch suspended in excess amount of water at ambient temperature can absorb up to 30% (by weight) of moisture (Jay-lin, 2003; Q. Liu, 2005). The water absorbed is usually present in the amorphous region of the granule and this process of hydration is reversible at ambient temperature (Jay-lin, 2003). Figure 4 shows that starch at 50^{o}C (at the start of region A) remains insoluble with low and unchanged viscosity. As the system continues to heat up from 50^{o}C to 65^{o}C (region A), the viscosity of the system remains unchanged.

2.4.4.3 Gelatinization

Figure 4 shows that at the “pasting temperature” or “onset temperature” of ~65^{o}C (Point B), the process of gelatinisation begins for rice starch. Gelatinization is defined as “the collapse or disruption of molecular orders within the starch granule manifested in irreversible changes in properties such as granular swelling, native crystalline melting, loss of birefringence, and starch solubilisation” (Mitolo, 2005). With continuous heating (Point B to C), several actions take place simultaneously: diffusion of water inside the starch granule with a limited swelling, disappearance of birefringence, loss of crystallinity of the granule, endothermal phase transitions, predominant swelling of the granule after the loss of birefringence, and a decrease in the relaxation times of the water molecules (Colonna & Buleon, 2009). The shear forces caused by the swollen rice starch granules required to squeeze past one another result in an increase in viscosity in the pasting curve (Point B to C). As the temperature increases further (Point C to D), more hydration and swelling occurs in the amorphous regions.

2.4.4.4 Pasting

Figure 4 shows that with continuous heating at 95^{o}C (close to Point D), the process of pasting takes place. This includes granular swelling, additional leaching of dissolved amylose molecules, and rupture of the fragile swollen granules. A peak viscosity (Point D) is generated at the equilibrium point between the swelling with amylose leaching (which causes viscosity to increase) and rupturing (which causes viscosity to decrease) (Mitolo, 2005).

2.4.4.5 Breakdown

Eventually, the fragile swollen granules “rupture” (thixotropic behaviour) under the shearing conditions and high holding temperature (95^{o}C). As a result, the viscosity decreases (Point D to E) to a holding strength point (Point E) to form a “hot paste” in Figure 4. A “hot paste” is composed of a continuous phase of primarily amylose and lower molecular weight amylopectin fractions; while the discontinuous phase made up fragments of swollen granules (Colonna & Buleon, 2009).

2.4.4.6 Retrogradation & gelation

As the system starts to cool the paste to 25^{o}C (Point E to F), the paste becomes more elastic and develops distinct solid-like properties, in which the final viscosity is obtained (Point F) in Figure 4. Pastes and molten materials are metastable non-equilibrium states and undergo structural transformations during cooling and storage (Colonna & Buleon, 2009). The linear amylose molecules form strong hydrogen bonds to create a three dimensional network that traps water and increases the immediate rigidity of the starch mass (A. Brown, 2008; Yook, Pek, & Park, 1993). Therefore, the initial gel formation in starch is dependent on the presence of sufficient levels of amylose. Upon storage for several days or weeks, the amylopectin gradually undergoes crystallization within the swollen granules and co-crystallization with amylose molecules, which results in an increase in the rigidity of the granules and reinforcement of the amylose matrix (Vasanthan, Li, Bressler, & Hoover, 2012). Therefore, amylose crystallization results in the initial development of gel firmness (final viscosity), while amylopectin crystallization results in an increment in gel firmness after few days or weeks of storage (Vasanthan et al., 2012).

2.4.5 Characterization techniques for starches

2.4.5.1 Rheological properties

The reference method for measuring the pasting properties of starches are based on two specialised equipment called Brabender Visco-Amylo-Graph (BVA) and the Rapid Visco Analyser (RVA) (Matignon et al., 2014). BVA and RVA measure the changes in consistency of a starch suspension under constant stirring and a heating and cooling cycle. However, the consistency is sometimes indecorously called

“viscosity” (Matignon et al., 2014). BVA and RVA are used worldwide with established methodologies, but they are not sensitive enough to measure low viscosities or minor changes in viscosity. In addition, these methods do not allow interpreting the structure of the starch paste/gel because of the unknown measurements conditions (Matignon et al., 2014). However, recent advances in technology have allowed for improved rheological measurements using instruments such as the rheometer. The starch cell in combination with the rheometer enables the easy analysis of starch pasting and gelling behaviour at the required heating and cooling rates in a rotational mode. This has provided unprecedented reproducibility for starch viscosity testing (Cho & Kang, 2011).

2.4.5.2 Thermal properties

Differential Scanning Calorimetry (DSC) analyses the difference in temperature between the sample and a reference material against time or temperature in a specified atmospheric condition. It specifically measures the amount of heat absorbed or released by a material (e.g. starch) undergoing chemical or physical changes such as gelatinisation (Jay-lin, 2003; Mohomed & Bohnsack, 2013). DSC can be used to characterise the changes in the gelatinisation temperature range (onset, peak, and end) of starch in the presence of spice / herbal extracts. The DSC generates a distinct endothermic peak(s) that can be used to determine the changes in the gelatinisation temperature range.

2.4.5.3 Structural properties

Microscopic techniques such as hot stage optical microscopy (HSOM) and scanning electron microscopy (SEM) are suitable method for analysing the morphological changes of starch granules (with or without spice / herbal extracts) during heating and cooling (Cai, Cai, Zhao, & Wei, 2014; Jay-lin, 2003). HSOM shows real-time granular swelling of starches that are usually larger. However, protein or non-starch polysaccharide molecules cannot be viewed under the HSOM. On the hand, SEM can intrinsically analyse structural characteristics of both smaller polymer (e.g. proteins) and larger structures (e.g. starch granules).

2.5 Starch-Spice/Herb Interactions and Complexes

The interaction between starch and components found in spices / herbs is the focus of this entire study. The only documented interaction reported in literature was between Mesona herb and starches, which is briefly reviewed in this section.

Mesona herb comprises of dried leaves and stems of the *Platostoma palustre* plant (Figure 5a and 5b). It is commonly known as “xian cao” (in Mandarin Chinese) and is widely consumed in South East Asia and East Asia in the form of medicine, herbal tea or as a gel-based dessert called ‘grass jelly’ (Figure 5c). The grass jelly dessert is a uniquely rigid gel that is made using Mesona herb and starch.

		
Figure 5a: *Fresh Mesona (Platostoma palustre) leaves*	***Figure 5b:*** *Mesona herb (dried Platostoma palustre leaves and stems)*	***Figure 5c:*** *Hard grass jelly, a dessert made from Mesona and starch mixture*

An ionic heteroglycan polysaccharide (Mesona gum) has been identified in the aqueous extract of the herb (Lai & Liao, 2002; Lai, Tung, & Lin, 2000). The Mesona gum solution has a low-viscosity in water with a pronounced shear-thinning characteristic. However, in the presence of starch and heat, it forms a rigid gel (Lai & Liao, 2002; Lai et al., 2000). Studies have proposed that the leached amylose molecules rearrange themselves with the Mesona gum molecules to form junction zones that result in a firm gel. The starch-gum interactions are primarily based on hydrogen bonds (Feng et al., 2014; Feng, Ye, Zhuang, Fang, & Chen, 2012). Figure 6 shows a schematic presentation of the proposed structure.

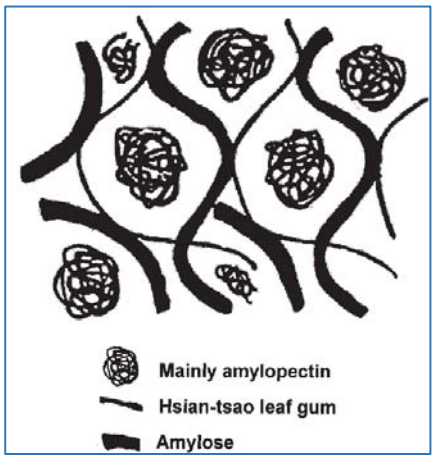


Figure 6: *A schematic presentation of the structure of starch-Mesona gel. Source: (Lai & Liao, 2002)*

In recent years, Mesona gum has been extensively studied for its ability to form a uniquely hard gel that can be sliced, shaped, or grated. Mesona gum has also been recently used to improve texture, retard staling, prolong the shelf-life, and enhance the gelatinization of wheat bread (M. Liu & Feng, 2014). The starch-Mesona gum interaction has also been effectively used as a fat substitute in Chinese sausages that allows better water-holding capacity and improves emulsion stability (Feng et al., 2013). Gels formed by Mesona gum and rice starch may potentially be used as a novel filler or thickener in the near future.

2.6 Starch-Saccharide Interactions and Complexes

The effects of monosaccharides, disaccharides, and non-starch polysaccharides on the pasting and thermal properties of native starches are reviewed in this section. These saccharides are naturally present in most spices and herbs and may independently influence the starch pasting properties.

2.6.1 Effect of simple sugars

Studies on the thermal properties have shown that the addition of simple sugars to various starches increased the onset temperature (initiation of gelatinization), peak temperature (peak of gelatinization), and end temperature (end of gelatinisation) (Ai & Jane, 2015; Perry & Donald, 2002; Sablani, 2009; W. Wang et al., 2015). The

temperature increased with increasing molecular weight and concentration of the sugar added to the system (Perry & Donald, 2002; Sablani, 2009).

Studies on the structural properties have shown that at low sugar concentration, the sugar molecules retarded the granular disintegration of starch and consequently increased the swelling ability of the granules. Generally, as the concentration of sugar increased, the swelling of starch granules increased as well (F. B. Ahmad & Williams, 1999; Hoover & Senanayake, 1996). However, after a certain sugar concentration point, the swelling of the starch granules decreased (F. B. Ahmad & Williams, 1999; Hoover & Senanayake, 1996). This concentration point ranges from 20-36% and may vary according to the types of starch or sugar used (F. B. Ahmad & Williams, 1999; Hoover & Senanayake, 1996).

Studies have also shown that the presence of sugar decreased the amount of amylose leached into the continuous phase, which resulted in a lower final viscosity and gel strength (F. B. Ahmad & Williams, 1999; Hoover & Senanayake, 1996). Therefore, the addition of sugars generally increases the peak viscosity and decreases the final viscosity of starches (Ai & Jane, 2015; Torley & van der Molen, 2005). However, others have reported that both peak and final viscosities were increased with increasing concentration of sucrose (W. Wang et al., 2015).

Several hypotheses have been proposed on the influence of sugars in stabilizing the structure of the starch granule and thereby delaying gelatinization (Biliaderis, 2009). Some of these include: (i) depression in water activity, (ii) direct sugar–starch interactions and (iii) anti-plasticization effect (Biliaderis, 2009).

In the (i) depression in water activity mechanism, it was proposed that competition for water between starch and sugar causes a reduction in the mobility of water and impedes the penetration of water into the granule, thus delaying gelatinization (Sablani, 2009). In the (ii) direct sugar-starch interaction mechanism, it was proposed that sugar molecules stabilize amorphous regions of starch by forming bridges with starch chains, thus increasing the energy required for starch gelatinization. It was also proposed that inclusion complexes are developed as sugar molecules penetrate into the interior of the starch molecule during swelling (Sablani,

2009). In the (iii) anti-plasticization mechanism, it was proposed that sugars act as an anti-plasticizing agent due to their higher molecular weight as compared to water (Perry & Donald, 2002). Therefore, the addition of sugar to starch-water system will reduce the level of solvent (water) plasticisation. As a result, a greater level of thermal energy input (higher temperatures) will be required before the starch granule could swell and begin to gelatinise (Perry & Donald, 2002).

2.6.2 Effect of non-starch polysaccharides

The interaction between starches and non-starch polysaccharides (NSPs) (e.g. xanthan gum, guar gum, and carrageenan) are being increasingly studied (K. K. T. Goh, Kumar, & Wong, 2014). However, there is rarely any literature available on the NSPs from different spices and herbs and their possible interactions with different starches. The only documented literature is about the NSP isolated from the Mesona herb and its profound interaction with different starches (*refer to Section 2.5*). Nevertheless, similar to Mesona herb, other spices and herbs may have functional NSPs that may potentially interact with starches.

The exact interaction between commercial NSPs and starches are not fully understood due to the complexity of such mixed systems (K. K. T. Goh et al., 2014). There are some proposed mechanisms for the interaction, which include: (i) NSPs influence on starch granular swelling, (ii) NSPs strengthening effect on starch granules, and (iii) synergistic interaction between starch and NSPs.

In the (i) starch granular swelling mechanism, it was proposed that the addition of certain NSPs, increases the viscosity of the system and causes a reduction in the mobility of water, which impedes the penetration of water into the starch granule. Consequently, the swelling of starch granules and amylose leaching are reduced, thereby reducing the peak and final viscosities of the starch (Bemiller, 2011; Funami et al., 2005; K. K. T. Goh et al., 2014).

In the (ii) strengthening of starch granules mechanism, it was proposed that NSPs such as xanthan gum and carrageenan could reduce starch granule disintegration and protect the granules against shear during processing (Appelqvist & Debet, 1997;

Bemiller, 2011). The reinforcement of starch granules was attributed to the formation of hydrogen bonds between NSP molecules and amylose within the swollen granules and/or when the amylose begins to leach out (Bemiller, 2011; K. K. T. Goh et al., 2014; H. Liu, Eskin, & Cui, 2003). Consequently, the peak viscosity and final viscosity may increase, while the rupturing of the starch may reduce.

In the (iii) synergistic interaction mechanism, it was proposed that the earlier onset of viscosity increase and the greater final viscosity observed in a starch pasting curve are due to possible interactions between starch molecules and NSPs through the formation of a network or cross-linkages (Bemiller, 2011; K. K. T. Goh et al., 2014). Certain NSPs such as ι-carrageenan molecules and Mesona gum molecules could interact with leached starch molecules to form a three-dimensional network structure with increased strength (Eidam, Kulicke, Kuhn, & Stute, 1995; Feng et al., 2014). Therefore, the peak and final viscosities of such systems are usually enhanced. In contrast, some NSPs can also interfere with intermolecular association among starch molecules in starch gel structures and reduce the viscosity (Bemiller, 2011; K. K. T. Goh et al., 2014).

2.7 Starch-Protein Interactions and Complexes

2.7.1 Proteins in spices and herbs

Proteins in spices and herbs are generally not isolated and characterised for its functionality or biological properties. In recent years, only a few bioactive proteins have been isolated from different spices and herbs. These include 30 KDa protein with anti-fungal properties extracted from Indian ginseng herb (*Withania somnifera*); 43 kDa protein with hepato-protective properties extracted from pigeon pea herb (*Cajanus indicus*); and 107 kDa protein with anti-calcifying properties extracted from carom (*Trachyspermum ammi*) (Dhar et al., 2012; Tanzeer Kaur et al., 2009; Sarkar & Sil, 2006). To date, there are no studies reporting on the interactions between starches and proteins isolated from spices or herbs. However, there are other studies illustrating the interactions between starches and proteins isolated from milk, fish, beef, pork, legumes, and cereals (Considine et al., 2011; Elgadir et al., 2012;

Ingrid & Debet, 1997; Jamilah et al., 2009). There are no clear trends on how protein influences starch pasting properties due to the structural differences of the protein molecules and inherent gel forming ability of some proteins.

A study reported that there was no interaction observed between maize starch and sodium caseinate (Kelly, Van Wagenberg, Latham, & Mitchell, 1995). Similarly, no significant interactions were observed for fish protein-starch and pork protein-starch systems (Appelqvist & Debet, 1997; Jamilah et al., 2009; J. Y. Li & Yeh, 2003). However, there are numerous studies, which reported either an increase or a decrease in viscosities of starches due to the presence of proteins.

2.7.2 Increment in viscosity

Studies have shown that there are interactions between sodium caseinate and starches such as wheat and potato that resulted in an increase in the apparent viscosity of the system during starch pasting (Considine et al., 2011; Doublier, Marzin, Visdeloup, & Lefebvre, 1994). The authors proposed that the increment in viscosity was due to thermodynamic incompatibility as sodium caseinate altered the swelling volume of starch, thereby affecting the rheological behaviour of the sodium caseinate-starch mixture (Doublier et al., 1994; Lelievre & Husbands, 1989).

Increasing the concentration of casein with maize starch has also shown to decrease the gelatinization temperature and increase the peak and final viscosities of the system (Goel, Singhal, & Kulkarni, 1999). The authors suggested that the increase in the peak and final viscosities were due to the proteins being saturated in the continuous phase of the starch gel, and thus the volume of the phase accessible to the proteins is reduced; this causes an increase in concentration in the continuous medium, thereby resulting in a high viscosity (Goel et al., 1999).

2.7.3 Decrement in viscosity

Studies have also shown that viscosity can be decreased due to the interaction between starch and protein (Appelqvist & Debet, 1997; A. C. Bertolini, Creamer, Eppink, & Boland, 2005). Low concentrations (2.5%) of sodium caseinate slightly

reduced the peak viscosity and increased peak viscosity temperatures of rice starch (Noisuwan, Bronlund, Wilkinson, & Hemar, 2008). Similarly, starch gelatinised with α-casein had a lower peak viscosity and was reported to reduce lower postprandial glucose peak in pigs (Kett et al., 2012). Other studies have also shown that sodium caseinate distinctively decreased the viscosity of modified starch (Colflo) and potato starch (Appelqvist & Debet, 1997; A. C. Bertolini et al., 2005).

In one particular study, the thermal and rheological properties of potato starch in the presence of peptide-rich food materials (such as beer yeast extract, bread yeast extract, wheat-brewed product, and whey protein hydrolysate) were studied. (Sakauchi et al., 2010). Peptide-rich food materials with high-charged amino acid content increased the gelatinization temperature, restricted granular swelling, and moderately reduced the viscosity of potato starch. The peak viscosity of potato starch was reduced approximately 3 times with the addition of 0.4% peptide-rich food materials (Sakauchi et al., 2010). The authors proposed that this behaviour of starch-peptide paste would be valuable for providing a basis for controlling the vaporization of water from starchy batter during frying in hot oil or reheating by microwave that give a dry texture (Sakauchi et al., 2010).

Different authors have proposed that the decrease in viscosity can be due to: (i) competition for water between the proteins and the starch granules, or (ii) protein molecules binding onto the surface of the starch granules (Considine et al., 2011; Noisuwan, Hemar, Bronlund, Wilkinson, & Williams, 2007).

In the (i) competition for water mechanism, it was proposed that the protein molecules actively compete for water with starch granules and delay the degree of swelling that causes a decrease in the viscosity (Considine et al., 2011; Noisuwan et al., 2007).

In the (ii) protein-starch binding mechanism, it was proposed that added protein molecules could adsorb onto starch granular surface through hydrophobic interactions with granule-associated lipids and/or proteins. For example, wheat starch granule surface contains 10 major protein groups ranging from 5 to 149 kDa, as well as several lipid components (Baldwin, 2001). Studies have shown these

granule-associated lipids and proteins (e.g. puroindoline proteins) were responsible for the adhesion of added soy protein fractions to the exterior of the starch granules (Ryan & Brewer, 2005a, 2005b). Therefore, it is plausible that the adsorption of milk or soy proteins onto starch granules is mediated by these indigenous granule-associated lipids and proteins (Ryan & Brewer, 2005a, 2005b). The removal of the granule-associated proteins from the surface of wheat starch was reported to cause a decrease in the binding of added soy proteins (Ryan & Brewer, 2006). Studies have further validated these observations as sodium caseinate and whey protein isolate have been reported to be adsorbed onto granular surface of rice starch and reduce its viscosity (Considine et al., 2011; Noisuwan, Hemar, Wilkinson, & Bronlund, 2011).

2.8 Starch-Phytochemical Interactions and Complexes

2.8.1 Classification of phytochemicals

Phytochemicals are non-nutritive components of food and include terpenoids, phenolic compounds, glucosinolates, betalains, chlorophylls, organic acids and amines (Ali-Reza, 2010). The consumption of plant foods rich in phytochemicals has been linked with reduced risk of chronic diseases such as cardiovascular, certain cancers, and type II diabetes (Fan Zhu, 2015).

Spices and herbs have high amount of different phytochemicals as compared to other foods. For example, Table 7 shows that culinary spices and herbs such as cloves, peppermint, star anise, oregano and sage have been ranked as foods with highest amount of polyphenols (Perez-Jimenez, Neveu, Vos, & Scalbert, 2010).

Table 7: *Polyphenol content of common foods*

Name of Spices / Herbs	Amount of polyphenols (mg per 100 g)
Cloves	15,188
Peppermint, dried	11,960
Star anise	5,460
Mexican oregano	2,319
Common sage, dried	1,207
Rosemary, dried	1,018
Common thyme, dried	878
Ginger, dried	202
Black grapes	169
Apple	136
Green tea	89
Cumin	55
Cinnamon	27
Pear	17

Source: (Perez-Jimenez et al., 2010)

Some of these phytochemicals have been reported to interact with starches. The subsequent sub-sections discusses about starch interaction with phytochemicals found in plants such as tea, cereals, pomegranate peel, Chinese hawthorn fruit, and Chinese gall. It also discusses about starch interaction with two specific groups of phytochemicals (phenolic compounds and terpenoids). There are insufficient literature available on the specific interactions between starches and spice or herbal extracts to be reviewed.

2.8.2 Starch-tea phytochemical interactions

In the early 1980s, scientists observed that starch digestibility was affected by tannins and other polyphenolic compounds found in tea. It was suggested that the legume (red bean, split yellow, bush bean and kidney bean) and potato starches had possible interactions with tea's tannic acid and catechin, which increased their resistance to α-amylase breakdown (Deshpande & Salunkhe, 1982). In recent years, studies have shown that a slow digestion property of high amylose starch cooked in tea polyphenols was observed in animal models (Chai, Wang, & Zhang, 2013). Tea extract inherently had inhibitory effect on the enzymatic activity of pancreatic α-amylase and glucosidase during starch digestion (R. Goh et al., 2015). However, it was proposed that the tea polyphenols may be acting as a regulator to the digestibility of high-amylose starches through hydrogen bond-mediated amylose−tea

polyphenol complexation rather than inhibitory effect alone (Chai et al., 2013). Therefore, the combination of tea polyphenols and specific starch could be employed to manipulate postprandial glycemic response, which would be beneficial for individuals with metabolic diseases such as type II diabetes (Fan Zhu, 2015).

2.8.3 Starch-cereal flavonoids interactions

Proanthocyanidins are flavonoids (polyphenolic compound) that are found in sorghum and was reported to interact strongly with the starch fraction to increase resistant starch formation and decrease the digestibility of starch (Frederico Barros, Awika, & Rooney, 2014; F. Barros et al., 2012; Dunn, Yang, Girard, Bean, & Awika, 2015). The interactions appeared to be specific to amylose and linear fragments of amylopectin, suggesting hydrophobic interactions (F. Barros et al., 2012). In addition, it was proposed that sorghum condensed tannins were more effective than monomeric sorghum polyphenols, in interacting with amylose possibly through hydrophobic and hydrogen bonding (F. Barros et al., 2012; Mkandawire et al., 2013). Therefore, high molecular weight polyphenols may provide new opportunities to produce functional ingredients that reduce caloric density of starch-containing products while providing added health benefits (Frederico Barros et al., 2014; F. Barros et al., 2012; Dunn et al., 2015).

In a recent application, another flavonoid called anthocyanin (from anthocyanin-rich black rice extract powder) was incorporated into bread dough. The digestion rates of bread with anthocyanin-rich black rice extract powder were found to be reduced by 12.8%, 14.1%, and 20.5% for bread with 1%, 2%, and 4% of extract powder, respectively (Sui, Zhang, & Zhou, 2016). The authors suggested that the fortification of anthocyanins into bread is an alternative way to produce functional bread with a lower digestion rate (Sui et al., 2016).

2.8.4 Starch-botanical extract interactions

The phytochemicals in the aqueous extracts of four plants were studied for their interaction with wheat starch (Fan Zhu, Cai, Sun, & Corke, 2009). The extracts of pomegranate peel, green tea, and Chinese gall increased the peak viscosity of wheat starch, while the extracts from pomegranate peel, green tea, Chinese hawthorn fruit, and Chinese gall decreased the final viscosity.

The authors reported that the phenolic compounds in the extracts often possessed hydroxyl and carboxyl groups, which could alter the pasting properties of starches by competing for water with starch through hydration (Fan Zhu et al., 2009). However, a more plausible explanation is that the addition of these plant extracts (high in phenolic acids) significantly lowered the pH of the starch–water suspension, which suggests that the altered pH of the suspension partially contributed to the alteration of wheat starch pasting properties (e.g. final viscosity). Besides the differing pH, another mechanism proposed was the interaction between the phytochemicals and the leached amylose at the hydrophobic regions and binding to the side chains of amylopectin through hydrogen bonding and van der Waals force, which might change the short-term retrogradation of starch (Fan Zhu et al., 2009).

2.8.5 Starch-pure phenolic compound interactions

Twenty-five different pure chemical-grade phenolic compounds [0.7% (w/w)] have been studied for their effects on the pasting properties of wheat starch [10.7% (w/w)] suspension (F. Zhu, Cai, Sun, & Corke, 2008). Eleven of these phenolic compounds (Table 8) are naturally found in various spices and herbs. These compounds had an influence on the pasting properties of wheat starch (Table 8).

Table 8: *Effect of eleven phenolic compounds on wheat starch pasting properties*

		Effect On Wheat Starch Pasting Properties		
Phytochemical	**Found In**	**Peak Viscosity**	**Holding Strength**	**Final Viscosity**
Trans-cinnamic acid	Cinnamon	Increase (by 37 RVU)	Decrease (by 85 RVU)	Decrease (by 131 RVU)
Quercetin	Neem	Increase (by 27 RVU)	Decrease (by 1 RVU)	Increase (by 32 RVU)
Gallic acid	Tea	Increase (by 23 RVU)	Decrease (by 80 RVU)	Decrease (by 145 RVU)
Coumarin	Bael	Increase (by 22 RVU)	Decrease (by 33 RVU)	Decrease (by 27 RVU)
Proanthocyanidins	Arjun Tree	Increase (by 22 RVU)	Decrease (by 26 RVU)	Decrease (by 28 RVU)
Ferulic acid	Asafoetida	Increase (by 22 RVU)	Decrease (by 71 RVU)	Decrease (by 136 RVU)
Rutin	Bael	Increase (by 22 RVU)	Decrease (by 1 RVU)	Increase (by 31 RVU)
Hesperidin	Valerian	Increase (by 16 RVU)	Increase (by 7 RVU)	Increase (by 13 RVU)
Trans-stilbene	Treebine	Increase (by 9 RVU)	Decrease (by 1 RVU)	Increase (by 12 RVU)
Catechin	Tea	Decrease (by 14 RVU)	Decrease (by 33 RVU)	Decrease (by 45 RVU)
Epicatechin	Tea	Decrease (by 10 RVU)	Decrease (by 33 RVU)	Decrease (by 40 RVU)

Source: (F. Zhu et al., 2008).

The authors suggested that the differences in the pasting properties of wheat starch treated with different types of phenolic compounds were possibly due to pH differences, unique structural features of the compounds, or changes in the water activity (F. Zhu et al., 2008). The structural features, which might include: (i) the types of head group, (ii) the number, type, and position of functional groups (such as methoxy and hydroxy groups), and (iii) chain length may potentially influence the pasting properties of starches. The authors suggested that the functional groups in these phenolic compounds might interact with amylose and amylopectin through hydrogen bonding and van de Waals forces. Besides the structural characteristics, phenolic compounds was proposed to compete with starch in binding water molecules to form their hydrated form and reduce the water activity of the system (F. Zhu et al., 2008).

2.8.6 Starch-pure monoterpenoids interaction

Beside the health benefits, phytochemicals such as the terpenoids group impart aroma or flavour to the spices and herbs. Monoterpenoids have also been specifically reported to form complexes with starches and influence its properties. The interactions between starch and monoterpenoid flavour compounds are of interest in connection with flavour retention, release, and encapsulation (Conde-Petit, Escher, & Nuessli, 2006). Table 9 shows a list of monoterpenoids that have been reported to form complexes with amylose.

Table 9: *Examples of monoterpenoids that form complexes with amylose*

Flavour	Molecular weight	Natural Sources
Fenchone	152	Fennel
Thymol	150	Thyme, Carom
Menthone	154	Mint, Gerniums
Camphor	152	Rosemary, Blue Basil
Geraniol	154	Lemon Grass
Carvone	150	Caraway, Spearmint, Dill

Source: (Conde-Petit et al., 2006)

These monoterpenoids compounds were suggested to be entrapped between the helixes in the starch crystal. Similar to the previous sections, the amylose fraction was suggested to be able to form helical inclusion complexes with phytochemical (Conde-Petit et al., 2006; Tietz, Buettner, & Conde-Petit, 2008a, 2008b). The binding of monoterpenoids to starch is known as “inclusion complex formation” through the hydrophobic bonding in the amylose helix and/or polar interaction that involve hydrogen bonds between hydroxyl groups of starch and the monoterpenoid (Błaszczak, Misharina, Fessas, Signorelli, & Górecki, 2013). The binding of low molecular compounds to starch may also be due to their non-specific adsorption to the starch powders or to granule agglomerates (Conde-Petit et al., 2006; Tietz et al., 2008b). The non-specific adsorption indicates that the compounds are non-specifically adsorbed (positively or negatively) when they are subjected in the interphase only to long-range coulombic interactions / electrostatic interaction (attraction or repulsion).

Similar to starch-tea and starch-flavonoids systems, the starch-monoterpenoids complexes show lower enzymatic hydrolysis rate compared to starch alone (Tietz et al., 2008a) The degradation of starch- monoterpenoids complexes seems to promote simultaneously a structure built up towards more stable crystallites, which can be assigned to an enzymatical annealing (Tietz et al., 2008a). For example, menthone provides aroma in peppermint and field mint. A higher viscosity was reported for the starch-menthone system as compared to the starch system without menthone upon α-amylase addition (Tietz et al., 2008a). It was hypothesised that menthone acts as a nucleation agent for inducing structure build-up of starch segments and hinders starch degradation (Tietz et al., 2008a).

2.9 Effect of Mineral Salts on Starch Pasting Properties

Mineral salts are known to independently influence starch pasting properties based on the ion type and concentration (Biliaderis, 2009). There are many mechanisms proposed for these effects but literature continuously shows contradictions (Biliaderis, 2009). For example, rheological studies have shown that addition of calcium chloride increased the viscosity of maize starch but reduced the viscosity of potato and sweet potato starches (Jyothi, Sasikiran, Sajeev, Revamma, & Moorthy, 2005; Moore, Tuschhoff, Hastings, & Schanefelt, 1984). Therefore, the effect of ionic agents on starch pasting properties can be more complicated and contradictory than other solutes such as sugars.

The common understanding of the interaction is that anions (e.g. chloride) generally have a greater effect on starch pasting than cations (e.g. sodium, iron and calcium) (Biliaderis, 2009; W. Wang et al., 2015). Ions that are larger in diameter (e.g. chloride) and weaker electric field intensity will tend to break the links between the molecules and increase the solubility of the molecules ("salting in"). However, ions with smaller diameter (e.g. potassium) and stronger electric field intensity or polyvalent ions will tend to protect the links between the molecules and decrease the solubility of molecules ("salting out"). The “salting out” ions are able to decrease the solubility, swelling power, transparency, and particle size of starch significantly, while the “salting in” ions will increase these properties. The “salting out” ions will also

increase the gelatinisation temperature, while “salting out” ions will decrease it (W. Wang et al., 2015).

A hypothesis was also proposed for the phenomena arising from the interactions between starch and salts (Oosten, 1982). Oosten hypothesis proposed that starch suspension has properties similar to a weak acid ion exchanger, which is capable of exchanging some of it protons of its alcoholic group for other cations when the circumstances are favourable. It was hypothesised based on some studies that cations tend to protect and stabilize the granule structure, while anions were gelatinizing agents that ruptured the hydrogen bonds (Oosten, 1982).

2.10 Effect of pH on Starch Pasting Properties

The pH of the starch system drastically influence the starch pasting properties. The starches at acidic pH may have a lower peak and final viscosities. The starch granules become fragile and breaks down rapidly in the presence of acid, which could result in a lower peak viscosity (H.-H. Wang, Sun, Zeng, & Lu, 2000). While, acid hydrolysis of the amylose molecules could occur and cause a weaker amylose network formation that results in a lower final viscosity during cooling (H.-H. Wang et al., 2000).

The starches at alkaline pH may have a higher peak viscosity and a slightly lower final viscosity. This could be attributed to the strengthening of bonding forces within the granules in the presence of Na^+ or OH^- ions (Bhattacharya & Corke, 1996). In addition, the anions (OH^-) may associate at specific sites in the starch and create a large hydration sphere that results in higher viscosity. However, the alkaline pH would not be able to sustain granular integrity for long, thus resulting in the rapid breakdown of the starch granules (Bhattacharya & Corke, 1996).

2.11 Influence of Spices and Herbs on Carbohydrate Digestion

2.11.1 Anti-enzymatic properties

International Diabetes Federation stated that diabetes is one of the leading causes of death in the world with 415 million people currently diagnosed with the disease. Diabetes is caused by decreased secretion or increased resistance of insulin, which may result in high postprandial glucose levels (Saha & Verma, 2012). One of the control measures for type II diabetes is the management of hyperglycaemia (high blood sugar) using commercial α-glucosidase and/or α-amylase inhibitor drugs such as acarbose, miglitol and voglibose (Derosa & Maffioli, 2012). α-glucosidase and pancreatic α-amylase are enzymes in the brush border of the small intestine that are responsible for the hydrolysis of carbohydrates (Helms & Quan, 2006).

In recent years, various spices and herbs (e.g. cinnamon, sage, rosemary, basil, parsley, chili, garlic, and onion) have been reported to contain natural α-glucosidase and/or α-amylase inhibitors (Cazzola, Camerotto, & Cestaro, 2011; Huerta, Mihalik, Beckett, Maitin, & Vattem, 2010; Ji, Xiao, Dong, Ma, & Ni, 2010; Kim, Im, & Yoon, 2015; Morikawa, 2007; Nasu, Miura, & Gomyo, 2005; Ranilla, Kwon, Apostolidis, & Shetty, 2010; Wongsa, Chaiwarit, & Zamaludien, 2012). There is a strong correlation between the enzyme inhibitory activities and the phytochemical content (e.g. phenolic compounds) of the spice or herb (Oboh & Ademosun, 2011; Saliu, Ademiluyi, Akinyemi, & Oboh, 2012). However, the exact mechanism of action between the phytochemical and the enzyme has not been clearly explained (R. Goh et al., 2015). Some studies suggested that phytochemicals have either a non-competitive inhibition (reduces the activity of the enzyme and binds equally well to the enzyme) or an uncompetitive inhibition (binds only to the complex formed between the enzyme and the substrate) (Shobana, Sreerama, & Malleshi, 2009). Other studies have suggested that, the phytochemicals may interact directly with the starch structure to make carbohydrates less accessible to the digestive enzymes.

However, the National Centre for Complementary and Integrative Health (NCCIH) of the U.S. National Institutes of Health stated that there are insufficient randomized-

controlled human trials to suggest that any herbal extracts or supplements can help prevent or manage type II diabetes effectively.

Carom, which is the primary focus of this study, is the only spice from the Apiaceae family reported to have 0% α-amylase inhibition while other spices (dill, fennel, caraway, coriander, and anise) had 10-32% inhibition rate. However, it was also observed that carom had a high 49% a-glucosidase inhibition rate as compared to other spices (Paliyath, Bakovic, & Shetty, 2011).

2.11.2 Digestion stimulating action

In Traditional Indian Medicine (Ayurveda), culinary spices and herbs such as carom, cumin, fennel, ginger, and pepper are used as natural digestive stimulants for the management of common digestive disorders such as indigestion (Deshmukh, 1980; Mukhram, Suryakant, Mohamed, Patki, & Rangesh, 2011; Saodekar & Anjikar, 1985). These spices have been reported to stimulate the endogenous digestive enzymes (salivary, gastric, pancreatic, and intestinal), increase the secretion of bile, and reduce the food transit time (Caballero, Finglas, & Toldrá, 2015; Deshmukh, 1980; Mukhram et al., 2011; Platel et al., 2002; Platel & Srinivasan, 2000a, 2004; Saodekar & Anjikar, 1985; Srinivasan, 2005a, 2005b).

The digestive stimulant actions of these spices have been studied using *in-vitro* and *in-vivo* models (Platel & Srinivasan, 2004; Prakash & Srinivasan, 2012; Srinivasan, 2005b). For example, spice such as red pepper, ginger, and mustard have been reported to enhance the secretion of saliva and increase the activity of salivary amylase (Glatzel, 1967; Platel & Srinivasan, 2004). While paprika, black pepper and cinnamon have been reported to increase gastric acid output, which indirectly plays a key role in the digestion of proteins. Spices such as turmeric, ginger, and fenugreek have been reported to stimulate bile acid production and secretion by the liver, which indirectly plays a key role in the digestion of lipids (Platel & Srinivasan, 2004). The secretions are possibly influenced by the nerve centres that are stimulated by certain chemical compounds or “irritants” in these spices (Platel & Srinivasan, 1996). The effect may also be due to the activation and release of endogenous secretagogues (a substance which promotes secretion) (Platel & Srinivasan, 2004).

Certain spices may have a direct influence on the enzyme activity itself. For example, enzyme activity studies have shown that spices such as fenugreek, asafoetida, and carom enhanced pancreatic lipase activities (Platel et al., 2002; Platel & Srinivasan, 1996, 2000a). While turmeric, ginger and cumin enhances pancreatic amylase, chymotrypsin, and trypsin activities (Platel et al., 2002; Platel & Srinivasan, 2000a). Ginger also enhanced the intestinal lipase activity, while carom and fennel enhanced intestinal amylase activity. However, the mechanism for the influence in enzyme activities are poorly understood.

Spices such as ginger, carom, cumin, and asafoetida shortened the food transit time by 24-31 per cent in animal model. This reduction in food transit time could probably be attributed to acceleration in the overall digestive process as a result of increased availability of digestive enzymes and of bile acids that facilitate fat digestion (Platel & Srinivasan, 2004).

Carom is valued for its antispasmodic, digestive stimulant, anti-microbial, and carminative properties in different traditional medicine systems. Until today, carom aqueous extract is prescribed to children and adults for common stomach ailment and indigestion. Dietary carom had stimulatory influence on bile secretion and pancreatic enzymes. The continued intake of carom in experimental rats have shown that bile flow increased by 20%, biliary solids by 41% and bile acid by 30%. In addition, the pancreatic lipase activity increased by 26%, pancreatic amylase by 9%, chymotrypsin by 5%, and trypsin by 48% (Platel & Srinivasan, 2000b, 2001, 2004; Srinivasan, 2005b). However, the intestinal lipase activity decreased by 16%, pancreatic amylase by 9%, and acid phosphatase by 27% with continuous intake of carom. Conversely, a one-time exposure or consumption at high dosage suggested that carom increased the activity of intestinal lipase, amylase, and phosphatase. There was also a 28% reduction in the food transit time (Platel & Srinivasan, 2000b, 2001, 2004; Srinivasan, 2005b).

2.11.3 Naturally-occurring amylases

Certain spices and herbs have been documented to have anti-amylase activities. On the contrary, some spices and herbs may have naturally-occurring α- and β-amylases that varies in the degree of activity (Nomura, Arai, & Shiomi, 1999). Table 10 summarises distribution of amylase activity in various spices.

Table 10: *Amylase activity found in various spices*

Name	**Amylase Activity** (units/g of spice)
Cinnamon	0.10
Clove	0.21
Ginger	0.24
Turmeric	0.29
Rosemary	0.35
Oregano	0.35
Mint	0.43
Sage	0.54
Thyme	0.56
Coriander Seed	0.56
Carom	1.30
Fennel	1.50
Cumin	1.72

Source: (Nomura et al., 1999)

These amylases aid the plant in seed germination, hydrolysis of raw starch and maturation of the fruits. High amylase activities have been reported in spices such cumin, fennel and carom (Nomura et al., 1999). Medium amylase activities are reported coriander seed, thyme, sage and mint (Nomura et al., 1999). The amylases in spices with high activities have been reported to reduce the viscosity of gelatinised starch and cause hydrolysis to produce reducing sugars (Nomura et al., 1999). The amylase isolated from cumin had optimum activity at 50 - 60^{o}C. The isolated enzymes were stable up to 70^{o}C and at pH 5 to 7 (Nomura et al., 1999). Amylases from different botanical, mammalian, or microbial sources can have varying thermal stability. To date, there are little literature available on the properties or thermal stability of amylases found in spices and herbs. The presence of thermal stable amylases in spices and herbs can potentially explain the digestive properties of certain spices and herbs, even after heat treatment (cooking).

CHAPTER 3: GENERAL MATERIALS AND METHODS

3.1 Introduction

Methodologies such as rheological measurements, differential scanning calorimetry measurements, size exclusion chromatography coupled with a multi-angle laser light scattering photometer, zeta potential measurements, hot-stage optical microscopy analysis, scanning electron microscopy analysis, *in-vitro* starch digestion, and enzymatic treatments were carried out throughout this study. This chapter reports on materials and methods that were commonly used in various experimental designs. Therefore, only the methodologies of rheological measurements, hot-stage optical microscopy analysis, and scanning electron microscopy analysis are discussed in this chapter. Subsequent chapters will contain detailed information about the other methodologies.

3.2 Materials

Native rice starch (Remy ODR6, Beneo GmbH, Germany) was the predominately-used starch for most of the experimental designs. Other starches (such as waxy rice starch, native maize starch, waxy maize starch, native wheat starch, native potato starch, native pea starch, native tapioca starch, glutinous rice flour, and sweet potato flour) were obtained from Roquette Frères (France) or Mustafa & Samsuddin Co Pte Ltd (Singapore).

Carom (Natco Foods Ltd., UK) was the predominately-used spice for most of the experimental designs. Thirty-six different spices and herbs were also used in the screening. The spices and herbs were obtained from Mohamed Mustafa & Samsuddin Co Pte Ltd (Singapore), Wan San Hoe Ptd Ltd (Malaysia), and Xi'an Hua Rui Co Ltd (China).

All experiments and extractions used Milli-Q water (Milli-Q® Integral Water Purification System, Merck Millipore Corporation, USA), unless specified. The chemical reagents (acids, alkalines, phytochemicals, and buffers) and enzymes used in the study were purchased from Sigma-Aldrich Corporation (USA), Merck

Millipore (USA), and Megazyme (Ireland). The acids and bases used to adjust the pH from 2 to 9 were 1M hydrochloric acid and 1M sodium hydroxide (Sigma-Aldrich Corporation., USA). The mineral salts used were calcium chloride, sodium chloride, sodium phosphate, magnesium chloride, ferrous gluconate, and potassium chloride (Merck Millipore., USA). The phytochemicals used were thymol (T0501), tannic acid (403040), ρ-cymene (C121452), limonene (218367), γ-terpinene (223190), saponins (84510), trans-anethole (800429) (Sigma-Aldrich Corporation., USA).

3.3 Rheological Measurements

Rheological technique was the main method employed throughout this entire study, in an attempt to characterise and understand the effects of spice and herbal extracts on the pasting properties of starches. The protocol for rheological measurement of starch samples was modified based on the procedure outlined by American Association of Cereal Chemists Standard Method 61-02.01 (Determination of the Pasting Properties of Rice) (AACC, 2000).

A standard amount of 30.0 g of sample consisting of 10.0% (w/w) starch, varying amount of Milli-Q water and an added component (e.g. spice / herbal extract) was usually used in most experimental designs. All the rheological measurements were carried out using a Paar Physica Rheometer MCR 301 (Anton-Paar, Graz, Austria) in rotational mode using ST24-2D geometry stirrer and CC27-SS cup (Anton-Paar, Graz, Austria). Figure 7 shows the parameters used for the measurements.

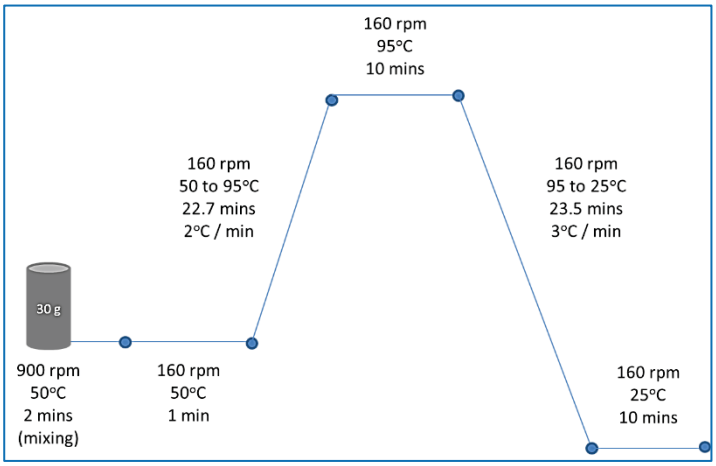


Figure 7: *Parameters and settings for the rheological measurement of starch systems using a Paar Physica Rheometer with a starch cell.*

30.0 g of sample was mixed for 2 minutes at 900 rpm and 50.0 ± 0.1ºC to prevent sedimentation. After the mixing, the data recording was initiated and the sample was rotated for 1 minute at 160 rpm and 50.0ºC ± 0.1ºC. The sample was then ramped from 50.0ºC to 95.0ºC (± 0.1 ºC) at 2.0ºC per min and held at 95.0ºC ± 0.1ºC for 10 mins at continuous stirring rate of 160 rpm. The cooked sample was then cooled from 95.0ºC to 25.0ºC (± 0.1 ºC) at 3.0ºC per min and held at 25.0ºC ± 0.1ºC for 10 mins at continuous stirring rate of 160 rpm.

3.4 Hot-Stage Optical Microscopy (HSOM) Analysis

Hot stage optical microscopy (HSOM) was used to observe the swelling of starch granules in the presence of carom extract during heating. The protocol for HSOM analysis was based on the procedure outlined by Cai et al. (2014). A starch-carom suspension (0.50 g) containing 1.5% (w/w) starch was prepared in an Eppendorf plastic tube (2 mL) and mixed thoroughly at 500 rpm for 1 minute using a vortex mixer (SA, Bibby Scientific Limited, UK). Approximately 200 µL of suspension was transferred from the tube onto a glass slide with a ring file reinforcement sticker, covered with a coverslip, and sealed with nail polish to prevent moisture loss during heating. The sealed specimen was then mounted on a hot stage apparatus (MP-10DMFH; Kitazato Co., Ltd., Fuji, Japan) and observed under a long focus M Plan Semi Apochromat objective (10× magnification) using a polarizing microscope (Olympus BX53, Olympus Optical Co Ltd, Tokyo, Japan). The hot stage was heated from 25ºC to 95ºC at a heating rate of 19ºC/min (± 0.5 ºC/min). Starch granular swelling was video-graphed under polarized light using a video camera (Obsolete, Olympus Corporation, Japan). Images of the starch granules were then retrieved at the respective temperatures of interest.

CHAPTER 4: SCREENING THE EFFECTS OF SPICE AND HERBAL EXTRACTS ON THE PASTING PROPERTIES OF RICE STARCH

4.1 Introduction

Rice is one of the most widely consumed staple food in the world. Various types of spices and herbs are commonly added to rice to incorporate colours, aromas, or flavours (Uhl, 2000b). However, the influence of these spices and herbs on the properties of rice starch has been poorly studied.

This study investigated the preliminary effects of thirty-six different spice and herbal aqueous extracts on the pasting properties of native rice starch. The pasting properties of native rice starch can easily be influenced by water activity, pH, temperature, and the presence of compounds such as proteins, polysaccharides, lipids, sugars, ions, enzymes, and phytochemicals. This study was the first of its kind to screen for any possible interactions or effects of common culinary and medicinal spice and herbal extracts on starch functionality.

4.2 Materials and Methods

4.2.1 Materials

Native rice starch was used in this study. Table 11 shows the thirty-six different spices and herbs used in the screening.

Table 11: *The types of spices and herbs used and their sources.*

<table>
<tr><th>Spice / Herb Name</th><th>Brand Name</th><th>Obtained From</th></tr>
<tr><td>Brahmi (Powdered)</td><td rowspan="4">(ARC Herbals., India)</td><td rowspan="32">Mustafa Centre (Mohamed Mustafa & Samsuddin Co Pte Ltd., Singapore).</td></tr>
<tr><td>Holy Basil (Powdered)</td></tr>
<tr><td>Indian Gooseberry (Powdered)</td></tr>
<tr><td>Neem (Powdered)</td></tr>
<tr><td>Black Tea (Dried)</td><td rowspan="3">(Lipton., UK)</td></tr>
<tr><td>Green Tea (Dried)</td></tr>
<tr><td>Oolong Tea (Dried)</td></tr>
<tr><td>Dill (Dried)</td><td rowspan="6">(MasterFoods., Australia)</td></tr>
<tr><td>Oregano (Dried)</td></tr>
<tr><td>Parsley (Dried)</td></tr>
<tr><td>Rosemary (Dried)</td></tr>
<tr><td>Sage (Dried)</td></tr>
<tr><td>Thyme (Dried)</td></tr>
<tr><td>Coriander Leaf (Dried)</td><td rowspan="2">(MDH Ltd., India)</td></tr>
<tr><td>Coriander Seed (Powdered)</td></tr>
<tr><td>Carom (Dried)</td><td rowspan="12">(Natco Foods Ltd., UK)</td></tr>
<tr><td>Cardamom (Dried)</td></tr>
<tr><td>Cinnamon (Dried)</td></tr>
<tr><td>Clove (Dried)</td></tr>
<tr><td>Cumin (Powdered)</td></tr>
<tr><td>Fennel (Powdered)</td></tr>
<tr><td>Ginger (Powdered)</td></tr>
<tr><td>Mint (Powdered)</td></tr>
<tr><td>Nutmeg (Powdered)</td></tr>
<tr><td>Star Anise (Powdered)</td></tr>
<tr><td>Turmeric (Powdered)</td></tr>
<tr><td>Coffee (Instant)</td><td>(Nescafé., Switzerland)</td></tr>
<tr><td>Black Pepper (Powdered)</td><td>(Ramdev., India)</td></tr>
<tr><td>Red Bush Tea (Dried)</td><td>(Twinings., UK)</td></tr>
<tr><td>Chinese Yam (Dried)</td><td rowspan="6">(Wan San Hoe., Malaysia)</td><td rowspan="6">Wan San Hoe Ptd Ltd., Penang (Malaysia)</td></tr>
<tr><td>Codonopsis Root (Dried)</td></tr>
<tr><td>Loguat Leaf (Dried)</td></tr>
<tr><td>Mulberry Leaf (Dried)</td></tr>
<tr><td>Perilla Leaf (Dried)</td></tr>
<tr><td>Reishi Mushroom (Dried)</td></tr>
<tr><td>Mesona Leaf (Dried)</td><td>(Xi'an Hua Rui., China)</td><td>Xi'an Hua Rui Co. Ltd.</td></tr>
</table>

4.2.2 Spice / herb extraction process

Figure 8 shows the extraction process used to obtain filtered aqueous extract of the thirty-six different spices and herbs.

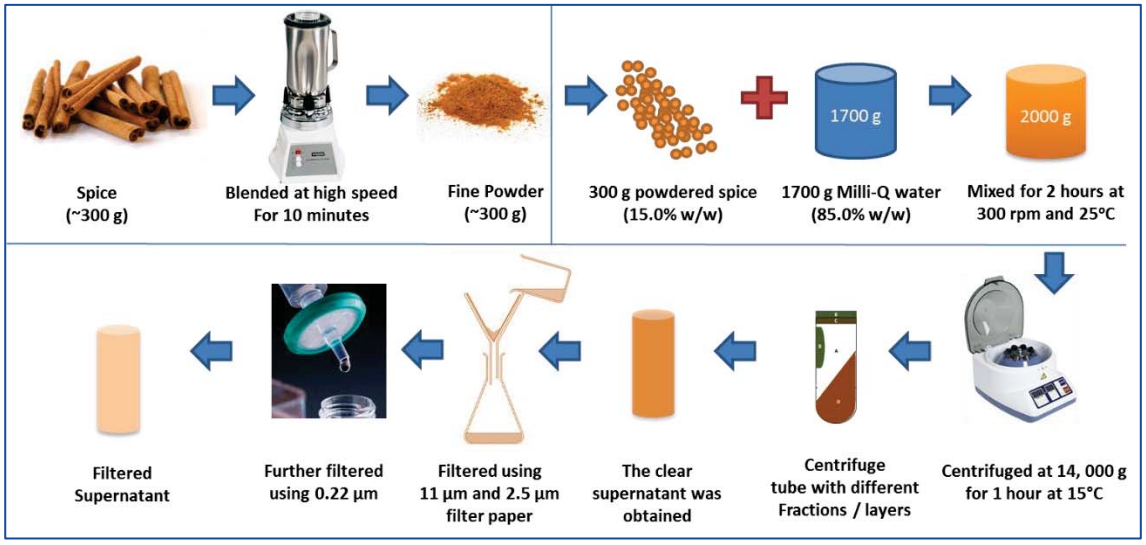


Figure 8: *Extraction process to obtain filtered aqueous extract (supernatant) of spices and herbs.*

Approximately 300 g of spice / herb were blended using a heavy-duty lab blender (Waring® Commercial, USA) at high speed for 10 minutes to obtain ~300 g of fine powder. A mixture of 15% (w/w) powdered spice / herb and 85% (w/w) Milli-Q water were mixed continuously at 300 rpm for 2 hours at 25^{o}C on a magnetic stirrer (Thermo Fisher Scientific Inc, USA). The mixture was then centrifuged at 14,000 g for 1 hour at 15^{o}C using benchtop centrifuge (Heraeus®, Thermo Fisher Scientific Inc, USA) fixed with a 50 mL rotor (HighConic™ 7500, Thermo Fisher Scientific Inc, USA). The clear supernatant obtained after centrifugation was filtered using a vacuum pump filtration system (Rocker 300, Rocker Scientific Co Ltd, Taiwan) with 11 µm and 2.5 µm filter paper (Whatman ® Qualitative Filter Paper, UK). The supernatant was then further filtered using a 0.22 µm filter membrane (Sartorius Minisart®, Germany). At the end of the process, a partially purified aqueous extract (supernatant) was obtained.

4.2.3 Sample preparation for rheological measurement

Figure 9 shows the sample preparation of the starch-spice / herb systems used for the rheological measurement.

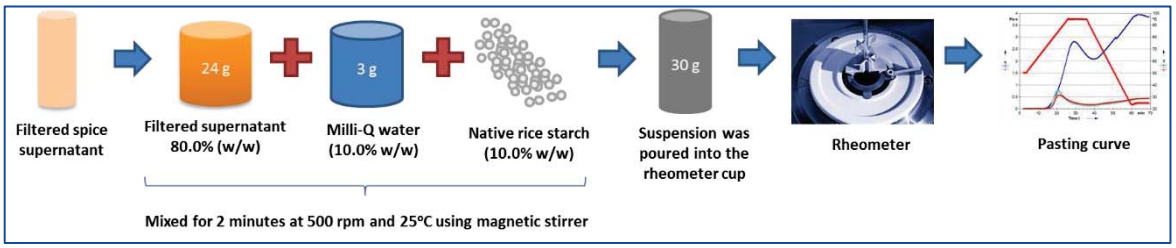


Figure 9: *Sample preparation procedure for rheological measurement*

Each sample (30.0 g) consisted of 80.0% (w/w) partially purified aqueous extract (supernatant) from a spice / herb, 10.0% (w/w) native rice starch, and 10.0% (w/w) Milli-Q water. The sample was mixed for 2 minutes at 500 rpm and 25°C using the magnetic stirrer. The pH of the system was then measured using a pH meter (Hanna Instruments, USA). The sample was then loaded into the CC27-SS cup for rheological measurement based on the parameters and methods described in *Section 3.3.* The changes in the viscosity of rice starch in the absence or presence of spice / herb aqueous extract were depicted in a pasting curve.

4.3 Results and Discussion

4.3.1 Influence on starch pasting properties

Table 12 shows the effects of thirty-six different spice and herb extracts on the peak viscosity of native rice starch. The spices and herbs are classified according to their family, genus, and species. Table 12 shows that twelve of the extracts had either peak viscosity suppressing or enhancing effects on rice starch.

Table 12: *Viscosity-suppressing and enhancing effects of 36 spice and herbal extracts on rice starch*

Common Name	Family	Binomial name	Effect on Peak Viscosity	pH[1] (± 0.05)
Carom	Apiaceae	*Trachyspermum ammi*	Suppressing	5.89
Coriander Seed	Apiaceae	*Coriandrum sativum*	Suppressing	5.58
Cumin	Apiaceae	*Cuminum cyminum*	Suppressing	5.68
Fennel	Apiaceae	*Foeniculum vulgare*	Suppressing	5.98
Coriander Leaf	Apiaceae	*Coriandrum sativum*	No effect	6.05
Dill	Apiaceae	*Anethum graveolens*	No effect	6.26
Parsley	Apiaceae	*Petroselinum crispum*	No effect	6.11
Codonopsis Root	Campanulaceae	*Codonopsis pilosula*	No effect	6.63
Chinese Yam	Dioscoreaceae	*Dioscorea polystachya*	No effect	6.62
Red Bush Tea	Fabaceae	*Aspalathus linearis*	No effect	6.02
Reishi Mushroom	Ganodermataceae	*Ganoderma lucidum*	No effect	6.32
Mesona	Lamiaceae	*Platostoma palustre*	Enhancing	6.52
Rosemary	Lamiaceae	*Rosmarinus officinalis*	Enhancing	6.19
Thyme	Lamiaceae	*Thymus vulgaris*	Enhancing	5.60
Holy Basil	Lamiaceae	*Ocimum tenuiflorum*	No effect	6.55
Perilla Leaf	Lamiaceae	*Perilla frutescens*	Suppressing	6.39
Mint	Lamiaceae	*Mentha spicata*	No effect	6.57
Oregano	Lamiaceae	*Origanum vulgare*	No effect	6.37
Sage	Lamiaceae	*Salvia officinalis*	No effect	5.75
Cinnamon	Lauraceae	*Cinnamomum cassia*	No effect	4.77
Neem	Meliaceae	*Azadirachta indica*	Suppressing	5.92
Mulberry Leaf	Moraceae	*Morus alba*	Suppressing	6.11
Clove	Myrtaceae	*Syzygium aromaticum*	Enhancing	4.20
Nutmeg	Myristicaceae	*Myristica fragrans*	No effect	4.80
Indian Gooseberry	Phyllanthaceae	*Phyllanthus emblica*	No effect	3.00
Black Pepper	Piperaceae	*Piper nigrum*	No effect	6.31
Brahmi	Plantaginaceae	*Bacopa monnieri*	No effect	6.05
Loguat Leaf	Rosaceae	*Eriobotrya japonica*	No effect	6.09
Coffee	Rubiaceae	*Coffea arabica*	No effect	6.32
Star Anise	Schisandraceae	*Illicium verum*	No effect	3.70
Black Tea	Theaceae	*Camellia sinensis*	No effect	6.33
Green Tea	Theaceae	*Camellia sinensis*	Enhancing	6.40
Oolong Tea	Theaceae	*Camellia sinensis*	No effect	6.20
Cardamom	Zingiberaceae	*Elettaria cardamomum*	No effect	6.23
Ginger	Zingiberaceae	*Zingiber officinale*	No effect	6.64
Turmeric	Zingiberaceae	*Curcuma longa*	No effect	6.33

[1] The pH value refers to the sample consisting of 80.0% (w/w) spice / herb aqueous extract, 10.0% (w/w) native rice starch, and 10.0% (w/w) Milli-Q water.
Note: The pH of the control sample comprising of 10% (w/w) native rice starch and 90% (w/w) Milli-Q water was 8.30 (+ 0.05).

Figure 10 shows that the aqueous extracts of seven spices and herbs (carom > cumin > fennel > mulberry leaf > perilla leaf > neem > coriander seed) exhibited viscosity-suppressing effects on rice starch.

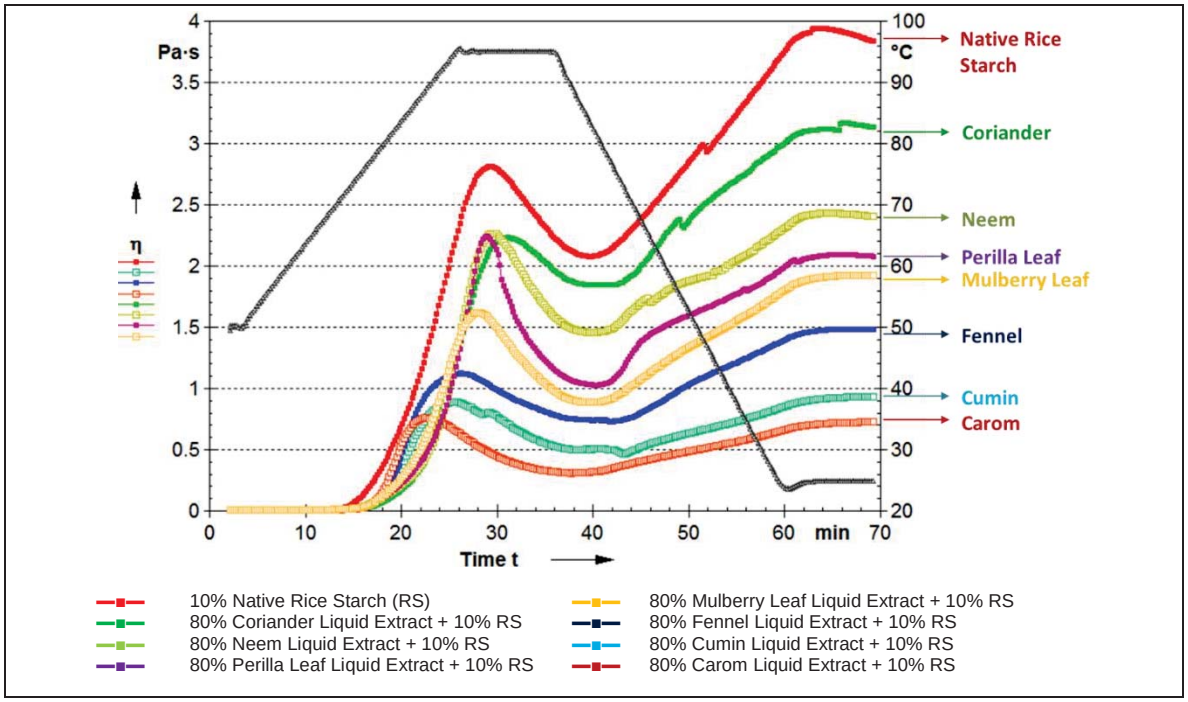


Figure 10: *The viscosity-suppressing effect of 80% (w/w) filtered liquid (aqueous) extracts of different spices and herbs on the pasting properties of 10% (w/w) native rice starch.*

The high precision of the rheometer and homogeneity of the aqueous samples provided data that superimposed upon replications (*refer to Figure A2 in Appendices for the superimposed data*). Therefore, duplicates were not carried out for the rheological data. Qualitative terminologies such as “viscosity suppressing” and “viscosity enhancing” were used in the description of the quantitative data observed in Figure 10.

The peak and final viscosities of the rice starches with carom, cumin, and fennel extracts were drastically suppressed as compared to the control (native rice starch suspension) (Figure 10). A moderate suppressing effect was observed for rice starches with white mulberry leaf, perilla leaf, neem leaf and coriander seed extracts (Figure 10).

On the other hand, Figure 11 shows that the extracts of five spices and herbs (mesona > rosemary > green tea > thyme > clove) enhanced the peak viscosity but

reduced the final viscosity of rice starch. The peak viscosity of the rice starch with mesona extract was drastically enhanced as compared to control (Figure 11). A slight enhancing effect of the peak viscosity was observed for rice starches with rosemary, green tea, thyme and clove extracts (Figure 11).

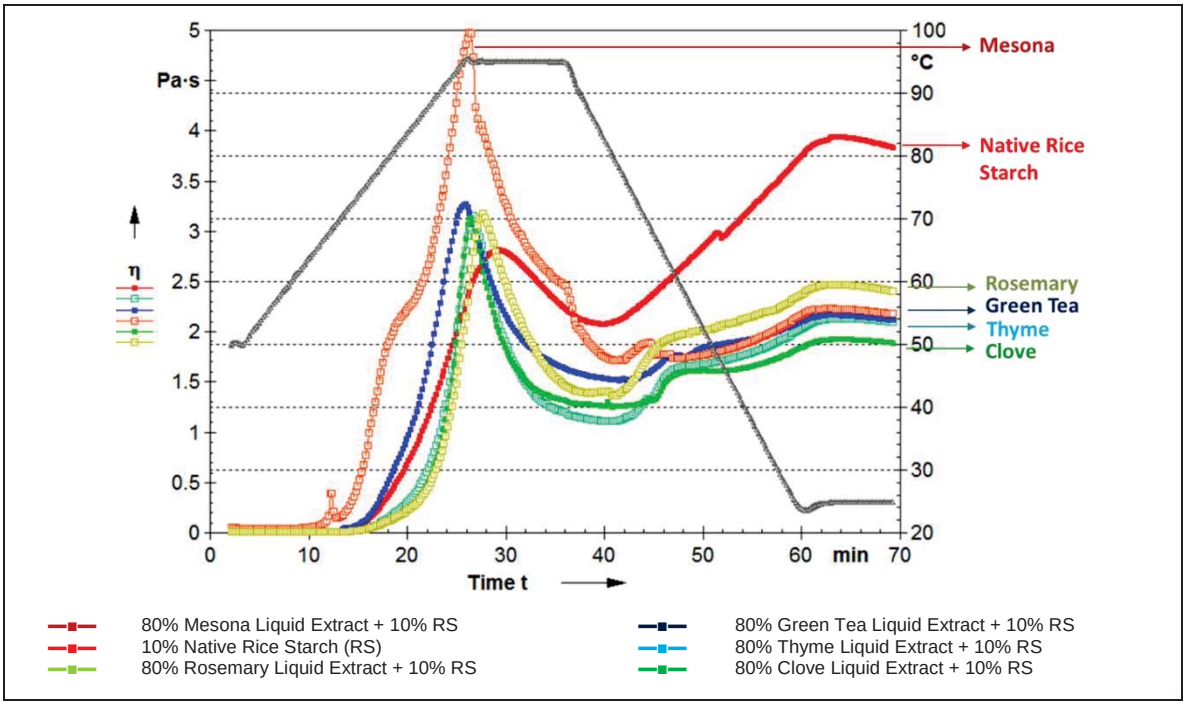


Figure 11: *The peak viscosity-enhancing effect of 80% (w/w) filtered liquid (aqueous) extracts of different spices and herbs on the pasting properties of 10% (w/w) native rice starch.*

There are insufficient literature available starch-spice interactions to explain the unique effects observed. The aqueous extracts of these twelve spices and herbs may contain proteins, polysaccharides, lipids, acids, bases, minerals, sugars, phytochemicals, or naturally occurring enzymes that may induce this viscosity-suppressing or enhancing effect in rice starch.

Based on the screening of the different spices and herbs, carom extract was found to have the highest viscosity supressing effect. To date, such a drastic viscosity suppression by a botanical extract has not been reported in other studies. Since the extract consisted of complex and unknown components, attempts were made to identify the key component(s) responsible for the viscosity suppressing effect. The possible components in carom extract giving this effect were investigated and discussed in subsequent chapters (*see Chapters 5 to 7*). A brief discussion on the possible causes for the suppression and enhancement effect of the remaining eleven spices and herbs are presented in this chapter.

4.3.2 Classification of spices and herbs

Table 12 shows that certain family and parts of plants had a profound effect on starch pasting properties as compared to others. Carom, cumin, fennel, coriander seeds, coriander leaf, parsley, and dill belong under the Apiaceae (Parsley) family. The viscosity-suppressing effect was only observed for the dried seeds or fruits (schizocarps) of the Apiaceae family (e.g. carom, cumin, fennel and coriander seeds) while the herbal leaves (e.g. dill, coriander leaf and parsley) had no effect (Table 12). Perilla leaf from the Lamiaceae (Mint) family had suppressing effect while mesona, rosemary, and thyme from the same family had an enhancing effect. The classification by family and the effect on viscosity showed no direct correlation, except for the trend seen in the dried fruits of the Apiaceae family.

4.3.3 Effect of macromolecules

Macromolecules such as proteins and polysaccharides are naturally present in spices and herbs. The identities and characteristics of most of these macromolecules are unknown. Studies have shown that certain proteins found in milk and non-starch polysaccharides found in other plants can induce a slight or moderate viscosity-suppressing or enhancing effects in the presence of starch (Appelqvist & Debet, 1997; Bemiller, 2011) (*refer to Section 2.6.2 and 2.7*). However, drastic suppression of viscosity like those observed in carom and cumin extracts (Figure 10) were uncommon in other studies.

Non-starch polysaccharides are known to affect starch pasting by influencing the granular swelling, strengthening the granules, or directly having a synergistic interaction with the starch molecules (K. K. T. Goh et al., 2014). Proteins are also known to affect starch pasting by competing with starch for water or by binding onto the surface of the starch granules (Considine et al., 2011; Noisuwan et al., 2007). These properties have shown to either increase or decrease the viscosity of starch.

Figure 11 shows that the aqueous extract of the Mesona herb (*Platostoma palustre*) significantly increased the peak viscosity of rice starch. It was also observed that a high concentration of Mesona herb extract on its own had a very low viscosity and remained unchanged during heating (data not shown). Studies have shown that a

unique non-starch polysaccharide (Mesona gum) has been identified in the aqueous extract of the Mesona herb and was responsible for the viscosity enhancing effect (Lai & Liao, 2002; Lai et al., 2000). During pasting, the leached amylose molecules from the starch granules are suggested to rearrange themselves with the Mesona gum molecules to form junction zones that result in a firm gel (Feng et al., 2014; Feng et al., 2012) (*refer to Section 2.5*).

To date, Mesona gum is the only polymer to be identified in an herb to show a synergistic interaction with starch. Apart from Mesona, other spices and herbs may have polymers that can potentially interact or influence starch pasting properties. However, there is a lack of clear evidence that polymers in other spices and herbs are the component responsible for the changes in the starch pasting properties.

4.3.4 Effect of phytochemicals

The twelve spices and herbs have varying types and quantities of phytochemicals (Table 1). A single spice or herb can have more than 100 phytochemicals in the system and any of these could potentially interact with starch. To date, only a few of these pure phytochemicals have been reported to influence the pasting and textural properties of starch paste and gel (Fan Zhu, 2015; F. Zhu et al., 2008). These phytochemicals include gallic acid, trans-cinnamic acid, ferulic acid, quercetin, rutin, hesperidin, catechin, epicatechin, coumarin, proanthocyanidins, tannins, and trans-stilbene (*refer to Section 2.8*).

For example, green tea chemical composition is complex as it is composed of proteins, cellulose, pectins, sugars, lipids, minerals, sterols, xanthic bases, flavonoids, chlorophyll, carotenoids, aldehydes, alcohols, esters, lactones, and hydrocarbons (Cabrera, Artacho, & Giménez, 2006). The list of phytochemicals in green tea is extensive. However, its main phytochemicals fall under the water-soluble flavonoid group called catechins, which constitute up to 30% of the dry leaf weight (Graham, 1992b). Other studies have reported that pure compounds of catechins and epicatechins caused a significant decrease in the peak and final viscosities of wheat starch (Graham, 1992a; F. Zhu et al., 2008) (Table 8). However, green tea extract used in this study was observed to increase the peak viscosity of

rice starch instead (Figure 11). Similarly, another study reported that pure compounds of quercetin that are commonly found in neem and dill, increased the peak and final viscosities of wheat starch (F. Zhu et al., 2008). However, neem extract used in this study decreased the peak viscosity of rice starch, while no effect was observed for dill extract (Figure 10).

The extensive list of phytochemicals in different spices and herbs increases the complexity and probabilities of interaction between starch and phytochemicals. It is probable that starch and phytochemicals interact to form inclusion amylose helices-phytochemical complexes mediated by hydrophobic bonds or hydrogen bonds (Fan Zhu, 2015). These complexes can either increase or decrease the viscosity of the system. However, due to the lack of clear evidence, it cannot be concluded that phytochemicals were solely responsible for the viscosity effects observed.

4.3.5 Effect of naturally-occurring amylases

Another plausible explanation for the observed viscosity-suppressing effect of carom, cumin, fennel, and coriander seed extracts (Figure 10) could be due to the presence of naturally occurring α- and β-amylases in the spices. A study reported high amylase activity in cumin, fennel, and carom, and medium amylase activity in coriander seed, thyme and sage (Nomura et al., 1999). These natural amylases were reported to reduce the viscosity of gelatinised starch by hydrolysing the polysaccharide chain to simples sugars (Nomura et al., 1999).

However, with the current knowledge, it is not possible to draw any conclusion whether amylases were involved in the viscosity reduction. The data reported thus far do not always show clear trends that spices containing high amylases would result in a high degree of viscosity suppression during gelatinisation of starches. Further work is needed to determine the activity of amylases in the spice and herbal extracts used in this study.

4.3.6 Effect of pH

The aqueous extract of spices and herbs contains varying amount of natural acids or bases. The effects of pH are known to influence starch pasting properties (W. Wang et al., 2015). The pH of 10% (w/w) native rice starch suspension was 8.30 (± 0.05). The addition of spice and herb extracts changed the pH of the rice starch system to values between 3.70 and 6.64 (± 0.05) (Table 1). There is a lack of clear evidence that pH was contributing to the viscosity suppressing or enhancing effect. For example, rice starch-carom system (pH of 5.89 ± 0.05) had a viscosity suppression, while rice starch-sage system (pH of 5.75 ± 0.05) with similar pH value had no effect on the viscosity (Table 1). Similarly, rice starch-rosemary system (pH of 6.19 ± 0.05) had peak viscosity enhancement but rice starch-cardamom system (pH of 6.23 ± 0.05) had no effect. With the current data, it is not possible to draw any conclusion that pH was involved in the viscosity reduction or enhancement.

4.4 Conclusion

Seven spices and herbs (carom, cumin, fennel, mulberry leaf, perilla leaf, neem and coriander seed) showed peak and final viscosity-suppressing effects, while five (mesona, rosemary, green tea, thyme and clove) showed peak viscosity-enhancing effects on 10% (w/w) native rice starch during starch pasting. Due to the complex composition of these extracts, further work is necessary to isolate key components such as proteins, polysaccharides, lipids, acids, bases, ions, sugars, phytochemicals, or naturally occurring amylases that may be involved in modifying the starch pasting properties. In the following chapter, attempts were made to evaluate individual components known to be present in carom or isolated from carom. Several key components from carom extract were individually evaluated with starch to observe the effects on rice starch pasting properties.

CHAPTER 5: THE EFFECT OF PARTIALLY PURIFIED CAROM EXTRACT ON THE PASTING, THERMAL, AND STRUCTURAL PROPERTIES OF RICE STARCH

5.1 Introduction

In the previous chapter, crude extracts of different spices and herbs were screened for their effects on starch pasting properties. Carom extract showed uniquely drastic peak and final viscosity-suppressing effect during starch pasting. In this chapter, carom extract was examined more thoroughly. Different fractions of carom that were separated during centrifugation were evaluated with starch to determine their influence on starch pasting properties. The chemical compositions of the clear supernatant fraction (partially purified carom extract) were determined. The effect of partially purified carom extract concentration on the pasting, thermal and structural properties of rice starch were investigated using rheometry, differential scanning calorimetry, and hot stage microscopy respectively.

5.2 Materials and Methods

5.2.1 Material

Carom and native rice starch were the primarily used in this study. Native potato starch was used in the structural study due to large granular size.

5.2.2 Carom extraction process

Figure 12 shows the extraction process used to obtain partially purified liquid carom extract. Similar carom extraction process has been reported by Hajare et al. (2005).

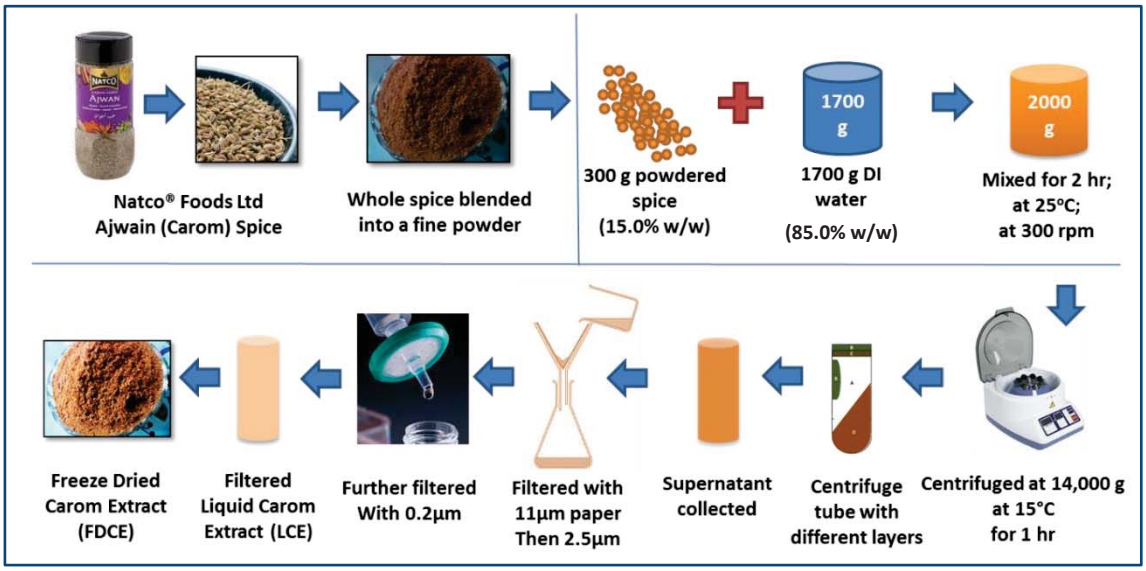


Figure 12: *Extraction process of carom to produce partially purified liquid carom extract and freeze-dried carom extract.*

Carom (500 g) was milled using a waring blender at high speed for 10 minutes to obtain a fine powder. The fine powder was stored at room temperature in an airtight container until use.

A mixture of 15% (w/w) powdered carom and 85% (w/w) Milli-Q water were mixed continuously at 300 rpm for 2 hours at 25°C on a magnetic stirrer. The mixture was then centrifuged at 14,000 g for 1 hour at 15°C using a benchtop centrifuge fixed with a 50 mL rotor. After centrifugation, different fractions (A, B, C, and D) of carom were obtained as shown in Figure 13.

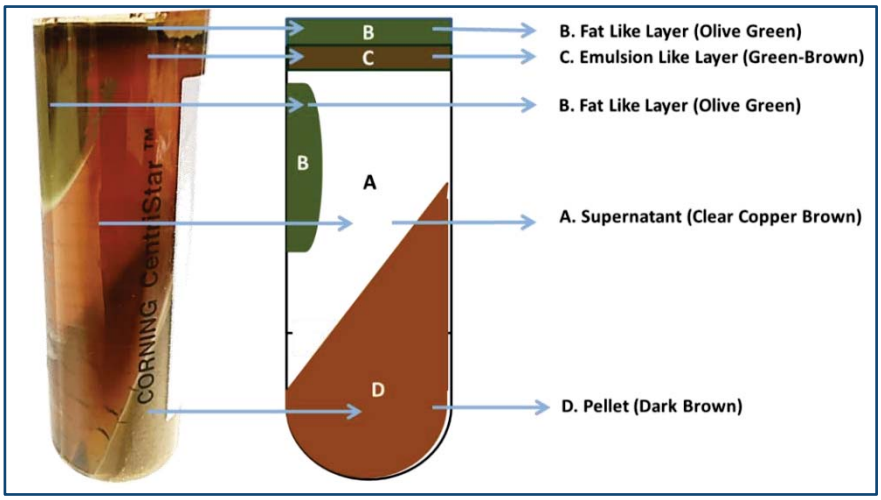


Figure 13: *The four different fractions obtained after the centrifugation of 15% (w/w) carom mixture at 14,000 g for 1 hour at 15°C.*

The supernatant (fraction A) obtained after centrifugation was filtered using a vacuum pump filtration system with 11µm and 2.5µm filter papers. The supernatant was then further filtered using a 0.22 µm filter membrane. After the filtration, a partially purified liquid carom extract was obtained. The extract was stored in Duran bottles and frozen at -20^{o}C until further use. The frozen extract was thawed at room temperature and mixed well before use.

5.2.3 Determination of the fraction responsible for viscosity suppression

After centrifugation, four different fractions of the carom were obtained (Fraction A, B, C, and D) as shown in Figure 13. Fraction A (clear copper-brown liquid) was the clear supernatant with most water-soluble components, Fraction B (fat-like olive green layer) was mostly fat-like components, Fraction C (emulsion-like green brown layer) was a mixture of component A and B, and Fraction D (solid dark brown layer) was mostly husk and insoluble components (Figure 13). Fraction D was discarded as waste.

The fraction with the highest viscosity suppression effect was studied using the rheometer. Samples (30.0 g) consisting of 80.0% (w/w) (Fraction A, B or C) was mixed with 10.0% (w/w) native rice starch and 10.0% (w/w) Milli-Q water. The samples were mixed for 2 minutes at 500 rpm and 25^{o}C using the magnetic stirrer. The sample was then loaded into the CC27-SS cup for rheological measurement based on the parameters and methods described in *Section 3.2.* The fraction with the maximum viscosity-suppressing effect was determined based on the pasting curve generated.

5.2.4 Chemical composition of freeze dried carom extract

A portion of the partially purified liquid carom extract (~300 g) was poured onto freeze-dryer trays (Scanvac Tray 3050, Denmark) to approximately 2 cm thickness. The samples were frozen at -20^{o}C for 3 hours. The trays containing the samples were then placed in the freeze-dryer chamber (LaboGene ApS Scanvac CoolSafe 95/55-80 Superior, Denmark). The sample underwent pre-freezing at -80^{o}C for 12 hours. The pressure was then adjusted to 0.5 mbar and the sample was freeze-dried

at −20 °C for 72 hours. At the end of the freeze-drying process, 300 g of liquid carom extract yielded ~35 g of freeze-dried carom extract.

The chemical composition of freeze-dried carom extract was determined by an accredited chemical laboratory (Nutritional Laboratory, Massey Institute of Food Science and Technology, Massey University) based on the AOAC methods. Table 13 shows the AOAC methods used in the determination of moisture, protein, lipid, dietary fibre, and ash contents. It also indicates the methodology employed to determine the total sugars, phenolic content, and mineral (calcium, potassium, sodium, phosphorus, iron, and magnesium) contents. The amount of carbohydrate was derived based on the difference between the sample dry weight and the total solids determined.

Table 13: *Methodology used for the chemical composition analysis*

Composition	Method
Moisture	AOAC 930.15 / 925.10
Protein	AOAC 968.06 (Leco)
Lipid	AOAC 954.02 (Mojonnier)
Total Dietary fibre	AOAC 991.43 (Megazyme)
Ash	AOAC 942.05
Carbohydrate	By Calculation (Difference)
Total sugars	Phenol Sulphuric Acid Method
Total Phenolic Content	Folim-Ciocalteau Method
Ca, K, Na, P, Fe, Mg	ICP Acid Digestion Method

5.2.5 Determination of the effect of carom concentration on pasting properties of rice starch

The effect of carom extract concentration on native rice starch pasting properties was determined using the rheometer. Each sample (30.0 g) consisted of freeze-dried carom extract [0.00, 0.02, 0.05, 0.28, 0.56, 1.10, 2.80, 3.80 and 4.70% (w/w)], 10.0% native rice starch, and remaining percentage of Milli-Q water. The samples were mixed for 2 minutes at 500 rpm and 25^{o}C using the magnetic stirrer. The sample was then loaded into the CC27-SS cup for rheological measurement based on the parameters and methods described in *Section 3.3*.

5.2.6 Determination of the effect of carom concentration on thermal properties of rice starch

Differential scanning calorimetry (DSC) was used to characterise the changes in the gelatinisation temperature range (onset, peak, and end) of native rice starch in the presence of varying concentrations of liquid carom extract. The protocol for thermal behaviour measurements using the DSC was modified based on the procedure outlined by Noisuwan et al. (2008). Thermal behaviour measurements were performed on a Q2000 MDSC (TA Instruments, New Castle, USA). Approximately 20 mg of samples consisting of 10% (w/w) native rice starch with varying concentration of liquid carom extract (0%, 1.6%, 8%, 16%, 32%, 80% and 90%) (w/w) were weighed into an empty stainless steel pans (TA Instruments, New Castle, USA). The pans were hermetically sealed and the samples were equilibrated at 20.0°C ± 0.1°C for 10 minutes and then a temperature ramp from 20.0 to 120.0°C was applied, at a heating rate of 5.0°C/min. The instrument was calibrated with indium (T_m=156.0°C, H=28.45 J/g) and an empty pan was used as a reference (Noisuwan et al., 2008). The onset temperature (T_{onset}), the peak temperature (T_{peak}), and end temperature (T_{end}) were calculated based on the distinct single endothermic peak obtained. The measurements were performed in duplicates and the results (onset, peak, and end temperatures) were presented as a mean of the two values obtained.

5.2.7 Determination of the effect of carom concentration on structural properties of potato starch

The effect of carom extract concentration on the swelling of native potato starch was studied using the hot stage optical microscopy. Native potato starch was used in the structural study due to large granular size. Each sample (0.5 g) consisted of freeze-dried carom extract [0.0%, 1.7%, 3.5%, 7.0% and 14.0% (w/w)], 1.5% native potato starch, and remaining percentage of Milli-Q water. The starch-carom suspension was prepared in an Eppendorf tube (2 mL) and mixed thoroughly at 500 rpm for 1 minute using a vortex mixer (SA, Bibby Scientific Limited, UK). The sample was then loaded onto the glass slides for measurement based on the parameters and methods described in *Section 3.4*. Images of the starch granules were then taken at 50, 72, 74, 76, 78, and 80°C.

5.3 Results and Discussion

5.3.1 Effect of different carom fractions on starch

Centrifuging the carom-water mixture yielded four fractions as shown in Figure 13. Figure 14 shows the effect of Fractions A, B, and C on starch pasting properties. Fraction D was discarded as waste due to its high insoluble material content.

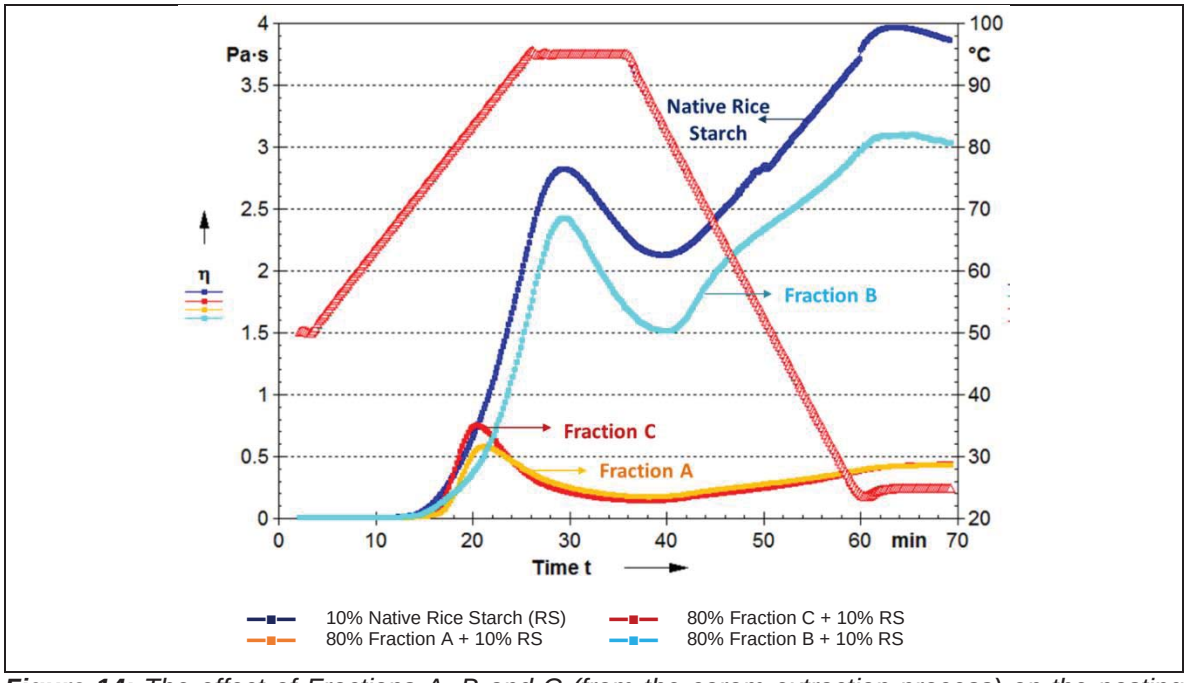


Figure 14: *The effect of Fractions A, B and C (from the carom extraction process) on the pasting properties of 10% (w/w) native rice starch (RS).*

Fraction A had the highest viscosity-suppressing effect. Fraction C had a similar effect to Fraction A, while Fraction B showed little suppression. It was evident that the water-soluble components (Fraction A) was responsible for the viscosity-suppression of rice starch. Fraction C had similar suppressing effect as A because it appeared to be predominately composed of Fraction A mixed with small quantity of Fraction B. Fraction B that appeared to be fat-like components did not influence the pasting properties of rice starch.

Studies have shown that water-soluble component of carom may contain proteins, non-starch polysaccharides, starch, acids, minerals, sugars, enzymes, uronic acid, and water-soluble phytochemicals (e.g. phytic acid, phenolic compounds, flavonoids, tannins and saponins) (Kaur & Arora, 2009; Pradeep et al., 1993). The water-soluble fraction may also contain small amount of fat-soluble phytochemicals

such as alkaloids and terpenoids (Ishikawa et al., 2001; Kaur & Arora, 2009). The phytochemical composition of Fraction A was not characterised in this study.

Fraction B that appeared like a fat layer may contain lipids and fat-soluble phytochemicals from the terpenoid group (e.g. thymol, γ-terpinene, ρ-cymene, β-pinene, myrcene, and carvacrol). Based on the pasting curves, it is reasonable to conclude that the lipids or fat-soluble phytochemicals in carom may not play a major role in the viscosity-suppressing effect on rice starch.

Therefore, as shown in Figure 12, Fraction A was filtered to obtain partially purified liquid carom extract and was further freeze-dried to obtain freeze-dried carom extract. Both the liquid extract and freeze-dried extract were used throughout the study to understand the water-soluble components inducing viscosity suppression.

5.3.2 Chemical composition of carom extract

The chemical composition of freeze-dried carom extract is shown in Table 14.

Table 14: *Chemical composition of freeze-dried carom extract*

Composition	Amount
Moisture	12.4%
Carbohydrates*	42.5%
- Sugars	15.3%
- Dietary Fibre	5.5%
Protein	23.2%
Fat	3.7%
Total Ash	18.1%
Total Phenolics	21.9 mg GAE/g
Potassium	6.50%
Calcium	1.83%
Magnesium	0.46%
Phosphorus	0.37%
Sodium	0.09%
Iron	0.0005%

Note: 857 g of liquid carom extract produced 100 g freeze dried carom extract
**By difference*

Based on the chemical composition (Table 14), proteins and presumably carbohydrates (based on the difference) appeared to be the main components present in the freeze-dried carom extract that could be influencing the pasting properties of starch. The high amounts of minerals, sugars, and phenolic compounds could also independently influence the pasting properties of starch (Appelqvist & Debet, 1997; Bemiller, 2011). Further experimental trials were carried out to investigate the role of each of these components in affecting starch pasting.

5.3.3 Effect of carom extract concentration

5.3.3.1 Rheological properties

Figure 15a shows the effect of different concentrations of freeze-dried carom extract on the rheological properties of rice starch. Figure 15b is a replot of Figure 15a, which shows the effect on peak viscosity.

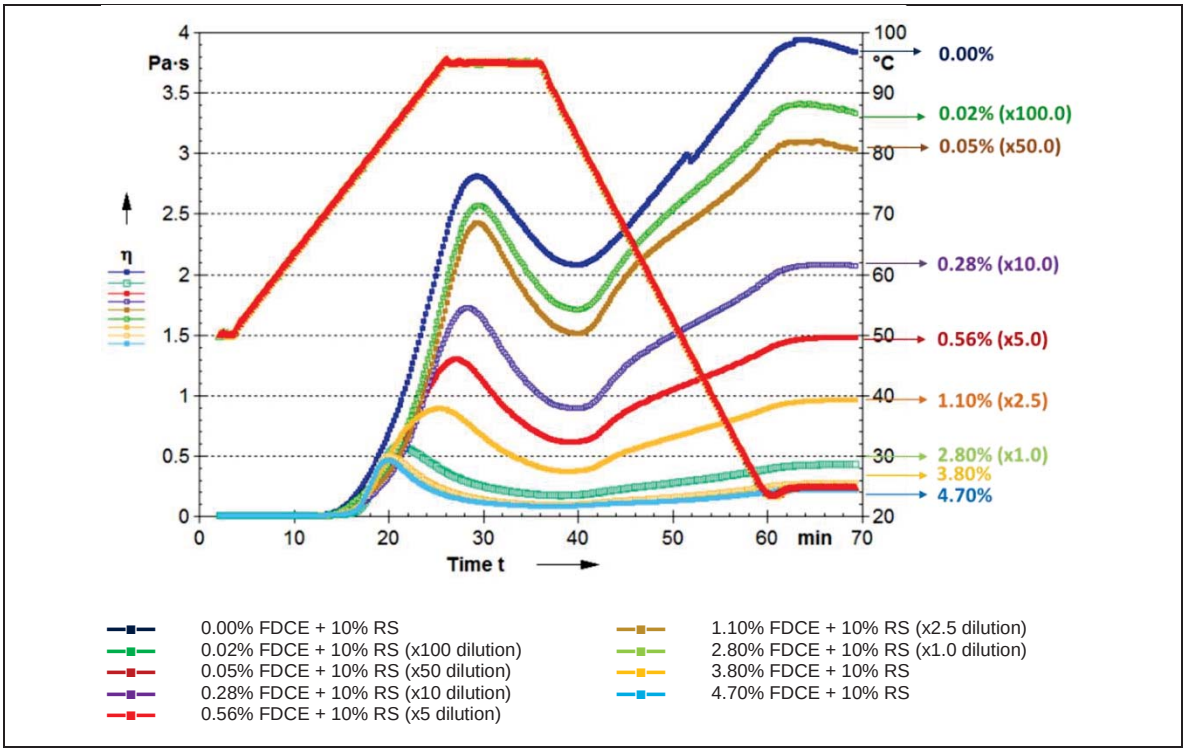


Figure 15a: *The effect of different concentrations of freeze-dried carom extract (FDCE) on the pasting properties of 10% (w/w) native rice starch (RS).*

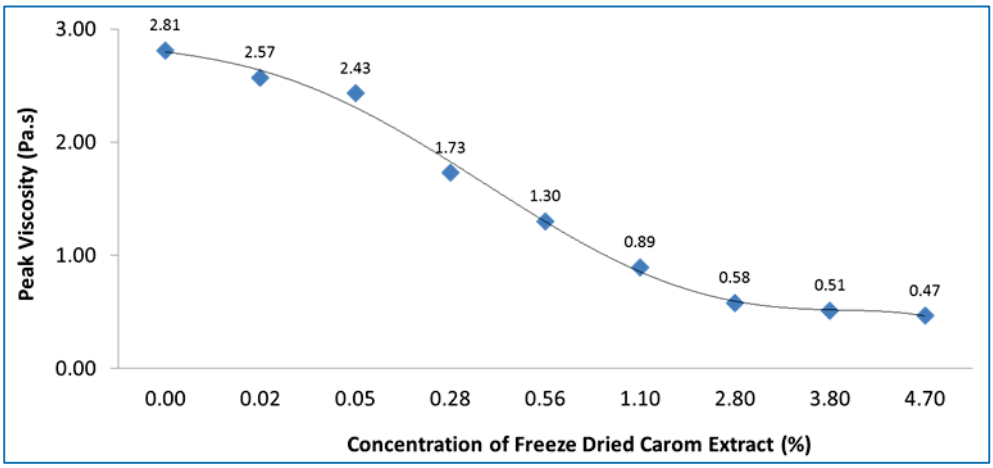


Figure 15b: *The effect of different concentrations of freeze-dried carom extract on the peak viscosity of 10% (w/w) native rice starch (RS).*

At low freeze-dried carom concentration [0.02% and 0.05% (w/w)], only a slight viscosity-suppressing effect was observed (Figure 15a and 15b). A more drastic suppression was observed at concentrations higher than 0.28% (w/w), with maximum effect at 4.80% (w/w). There was no drastic decrease in the viscosity between 2.80% and 4.80% (w/w) (Figure 15b). The viscosity of 0.02% to 4.70% (w/w) carom extract solution alone (without starch) was close to that of water (data not shown).

5.3.3.2 Thermal properties

Thermal studies on starch gelatinization were conducted using differential scanning calorimetry (DSC). The DSC generated endothermic curves consisting of onset, peak, and end temperature points for 10% (w/w) rice starch in excess amount of water or liquid carom extract. These points can be defined as the initiation of gelatinization (onset temperature), peak of gelatinization (peak temperature), and end of gelatinisation (end temperature) respectively (M. A. Rao et al., 2012). Figure 16 is a replot of the temperature points obtained from the DSC endothermic curves (*refer to Figure A3 in the Appendices*). Figure 16 shows the effect of increasing concentration of liquid carom extract on the thermal properties of rice starch.

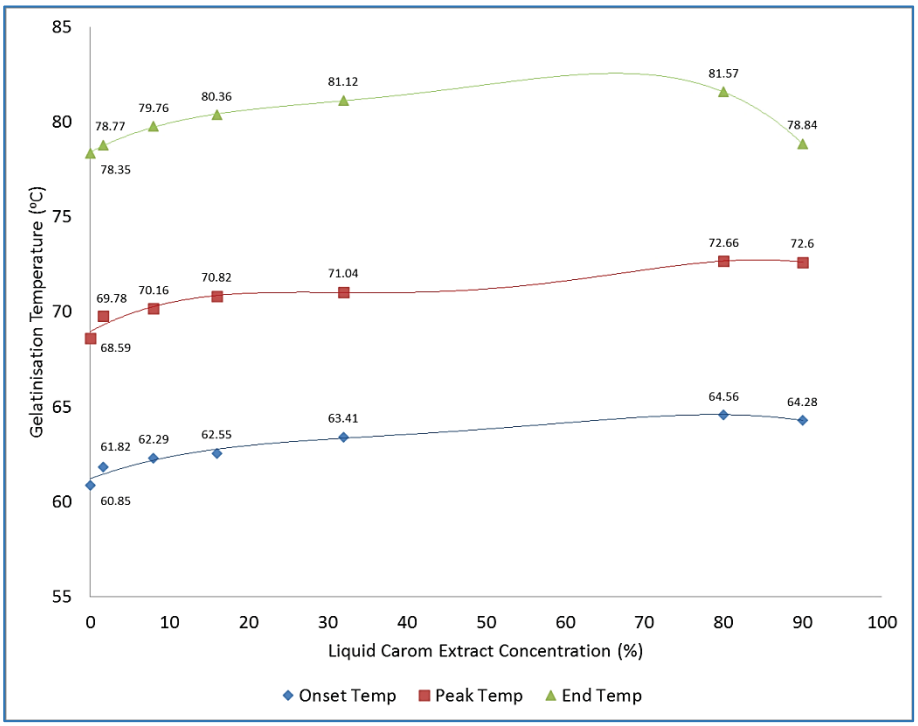


Figure 16: *Thermal properties of 10% (w/w) native rice starch at varying liquid carom extract concentration (0%, 1.6%, 8%, 16%, 32%, 80% and 90%) (w/w) analysed using the DSC.*

From the graph (Figure 16), the onset and end temperature of 10% (w/w) rice starch without carom extract were 60.85ºC and 78.35ºC respectively, with an interval of 17.50ºC. Other studies have reported similar values of 61ºC (onset) and 80ºC (end) for native rice starches (Collado & Corke, 2003; S Damodaran, K L Parkin, & O R Fennema, 2008; Henry & Alistair, 2006).

The onset, peak, and end temperatures of rice starch increased with increasing carom extract concentration (Figure 16). The onset and end temperature of 10% (w/w) rice starch with 80% (w/w) liquid carom extract were 64.56ºC and 81.57ºC respectively, with an interval of 17.01ºC. The addition of 80% (w/w) carom extract has increased the onset, peak, and end temperatures of rice starch by 3.71ºC, 4.07ºC and 3.22ºC respectively (Figure 16). However, the interval has remained relatively similar.

The results indicated that the gelatinisation process was not prolonged but rather delayed. The reason for the delay in gelatinisation (or a higher heat energy required for gelatinisation to occur) in the presence of carom extract was not entirely clear. Some reasons could be due to the presence of macromolecules or small molecular

compounds (e.g. sugars, salts, and phytochemicals) in the carom extract, which could increase the onset temperature and cause a delay in the initiation of gelatinization.

Thermal studies have shown that the addition of sugars to starch will usually increase the gelatinisation temperature range (Ai & Jane, 2015; Perry & Donald, 2002; Ratnayake, Otani, & Jackson, 2009). The temperature required for gelatinisation rises with increasing molecular weight and concentration of the sugar in the system (Perry & Donald, 2002; Sablani, 2009). A study has shown that 2% (w/w) sucrose added to starch resulted in an increase in the gelatinisation temperature by only 1.24^{o}C (Ratnayake et al., 2009). However, Figure 16 shows an increment of 3 - 4^{o}C for rice starch sample with 80% carom extract [which contains approximately 0.4% (w/w) total sugars (Table 14)]. The amount of sugar was too low in the carom extract to cause a drastic increment in the onset temperature observed in Figure 16.

Similarly, some salts (e.g. sodium chloride) are also known to increase the starch gelatinization temperature at low concentrations, while some other salts (calcium chloride) depress the gelatinisation temperature (Chiotelli, Pilosio, & Le Meste, 2002; Ratnayake et al., 2009). However, it is unlikely that the amount of sodium and other minerals (Table 14) was the probable cause for increment in the gelatinisation temperature.

Protein is the major component in carom extract (Table 14). Some proteins are known to increase the onset, peak and end temperature of starches (Considine et al., 2011; Elgadir et al., 2012; Ingrid & Debet, 1997; Jamilah et al., 2009). A DSC study showed that 10% (w/w) sodium caseinate with 10% (w/w) waxy rice starch, increased the onset and peak temperatures by 1^{o}C and 2^{o}C respectively. (Considine et al., 2011; Noisuwan et al., 2008). Therefore, the presence of proteins in carom extract may cause a slight increment in the gelatinisation temperature.

The presence of protein, sugars, salts, and/or many other compounds may synergistically or independently increase the gelatinization temperature of rice starch. However, with the current data, it is not possible to draw any conclusion for the increment.

The onset and peak temperatures observed in the pasting curve (Figure 15a) and DSC graph (Figure 16) differed. For example, 10% rice starch had an onset temperature of 65.0ºC (rheology) and 60.85ºC (DSC), and a peak temperature of 95.0ºC (rheology) and 68.59ºC (DSC). This could be due to the slower rate of heat transfer to the sample in the rheometer cup, as compared to the rapid heat transfer in the DSC. In addition, rheological measurements are performed under shear whereas there is no shear applied during a DSC measurement (Noisuwan et al., 2008). The peak temperature measured by DSC relates to the point during the heating of the starch where the maximum rate of gelatinisation occurred. Conversely, the peak viscosity temperature as measured by the rheometer relates to the equilibrium point between the starch granular swelling with amylose leaching (which causes viscosity to increase) and rupturing (which causes viscosity to decrease) (Mitolo, 2005). Therefore, it is reasonable not to expect that the DSC peak temperature and peak viscosity temperature from rheological data will be well correlated.

5.3.3.3 Structural properties

Figure 17 shows the effect of increasing concentration of freeze-dried carom extract on the structural properties of native potato starch using hot-stage optical microscopy. Figure 17 shows that most of the starch granules with 0% carom extract started swelling at ~72ºC and were completely swollen at ~76ºC. It is to be noted that the temperature applied to the minute quantity of sample in the hot stage microscopy did not represent accurately the temperature of the sample due to heat losses and a rapid heating rate (19°C/min). For instance, swelling was only observed at ~72°C by the hot-stage microscopy when the reported gelatinization temperatures for potato starches range from 56 - 69ºC (Table 6). However, the relative changes observed with temperature, accurately show the concentration effect on the swelling process.

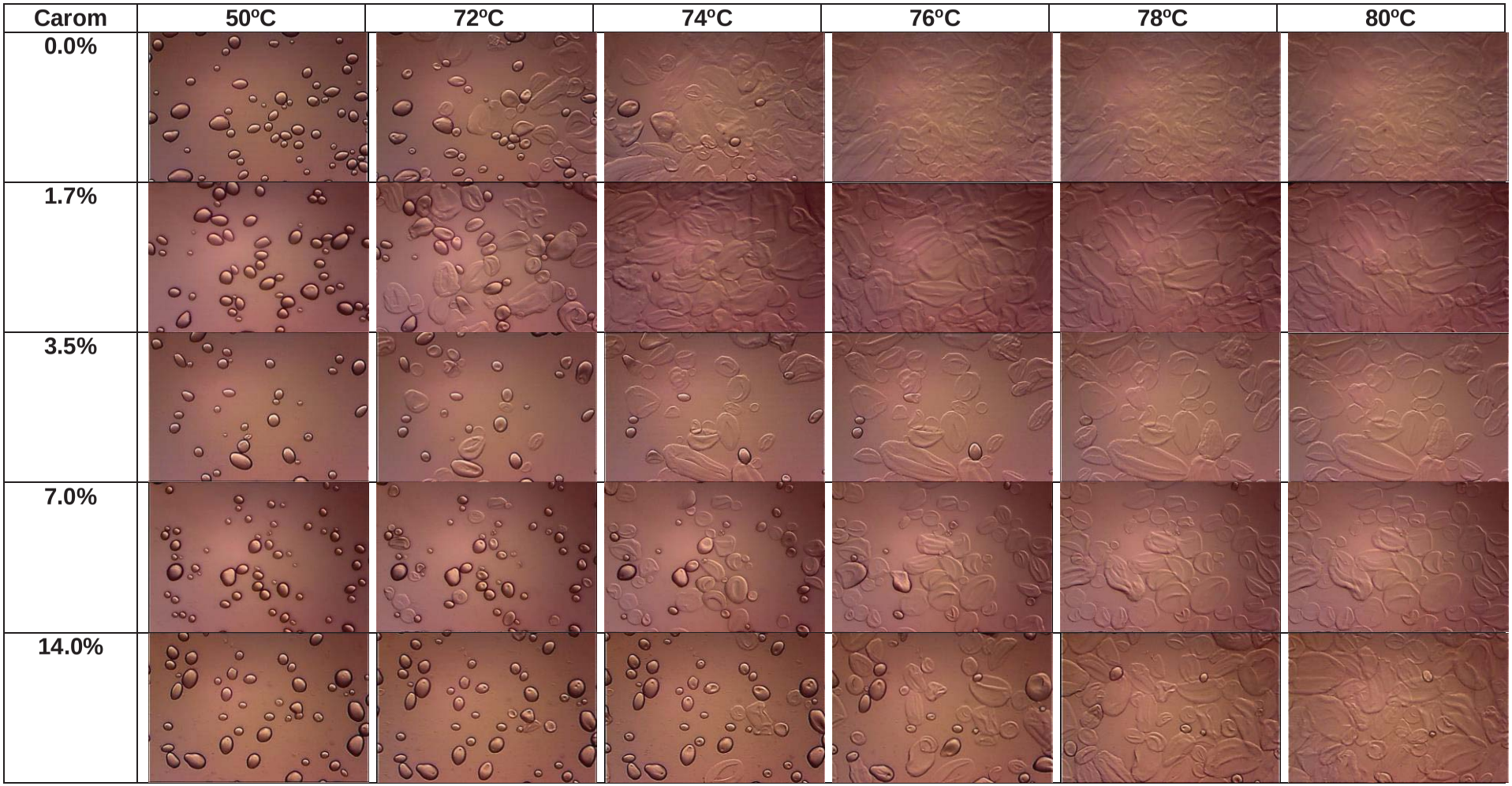


Figure 17: *Hot stage micrographs of 1.5% (w/w) potato starch with 0.0, 1.7, 3.5, 7.0 and 14.0% (w/w) freeze-dried carom extract dispersed in water was heated from 50°C to 90°C at the rate of 19°C/min.*

Based on the micrographs shown in Figure 17, when the concentration of carom increased in the starch system, the granular swelling was delayed. Most of the starch granules with 14% carom extract started swelling at ~76^{o}C and were completely swollen at ~80^{o}C. The micrographs of the structural study correlated well with the thermal and rheological studies in that the onset temperature (initialisation of gelatinisation) of starch was delayed in the presence of carom extract. The restriction of granular swelling could explain the low peak viscosity observed in the rheological data. However, all the starch granules eventually swelled completely at 90^{o}C (data not shown) so the decrease in the peak and final viscosities in the rheological data could be related to other interactions between starch and carom. The carom extract may be interfering with the amylose leaching and network formation during the cooling phase that resulted in a low final viscosity.

5.4 Conclusion

Fraction A containing water-soluble components was the main fraction in the carom extract that showed a viscosity-suppressing effect during starch pasting. The exact component(s) that caused this effect is currently unclear but it is likely to be the polymeric fractions in the carom extract. The carom extract was majorly composed of 42.5% carbohydrates (by difference), 23.2% protein, 18.1% ash, and 15.3% sugars.

Rheological studies showed that the peak and final viscosities of rice starch decreased with increasing carom concentration. Thermal studies using DSC showed increased onset, peak and end temperatures with increasing carom concentration. Observation of hot stage optical micrographs showed that the granular swelling of potato starch was restricted and delayed with increasing carom concentration.

CHAPTER 6: THE EFFECT OF SMALL MOLECULAR COMPOUNDS ON THE PASTING PROPERTIES OF RICE STARCH

6.1 Introduction

In the previous chapter, the basic chemical composition of partially purified carom extract and its effects on the rheological, thermal, and structural properties of rice starch were studied. The study showed that with increasing concentration of carom extract, the viscosity-suppressing effect and gelatinization temperature range (onset, peak, and end) increased, while the granular swelling of starch was delayed. In this chapter, the role of small molecular compounds (e.g. acids, bases, mineral salts, and phytochemicals) in inducing the viscosity-suppressing effect on native rice starch was studied. Some of these small molecular compounds have been reported to influence pasting properties of different starches. However, most of the compounds used here have not been extensively reviewed, especially in a rice starch system.

6.2 Materials and Methods

6.2.1 Materials

Native rice starch was the primary starch used in this study. The acids and bases used to adjust the pH (2 – 9) were 1M hydrochloric acid and 1M sodium hydroxide (Sigma-Aldrich Corporation, USA). The mineral salts used were calcium chloride, sodium chloride, sodium phosphate, magnesium chloride, ferrous gluconate, and potassium chloride (Merck Millipore, USA). The phytochemicals used were thymol (T0501), tannic acid (403040), ρ-cymene (C121452), limonene (218367), γ-terpinene (223190), saponins (84510), trans-anethole (800429) (Sigma-Aldrich Corporation, USA).

6.2.2 Determination of the effect of pH on rice starch

The effect of pH on rice starch pasting properties was determined using rheometry. Each sample (30.0 g) consisted of 10.0% (w/w) native rice starch, a specific quantity of 1M hydrochloric acid or 1M sodium hydroxide (according to the pH), and remaining percentage with Milli-Q water. The samples were mixed for 2 minutes at 500 rpm and 25^{o}C using the magnetic stirrer. The final pH of the different starch suspension samples were 2.00, 3.00, 4.00, 5.00, 6.00, 7.00, 8.00 and 9.00 ($\pm$ 0.05). The samples were then loaded into the CC27-SS cup for rheological measurement based on the parameters and methods described in *Section 3.3*.

6.2.3 Determination of the effect of mineral salts on rice starch

The effect of mineral salts (calcium chloride, sodium chloride, sodium phosphate, magnesium chloride, and potassium chloride) on native rice starch pasting properties was determined using rheometry.

0.5 M and 1.0 M of calcium chloride solution, sodium chloride solution, sodium phosphate solution, and potassium chloride solution; 0.1 M of ferrous gluconate; and 0.1 M and 0.5 M of magnesium chloride solution were prepared. Each salt was dissolved in Milli-Q water for 15 minutes at 500 rpm and 25^{o}C using the magnetic stirrer. A higher concentration of salt solution were prepared as compared to the typically amount found in carom extract, in an attempt to study the extreme effects of these salts on starch pasting properties.

Each sample (30.0 g) for rheological test consisted of 10.0% (w/w) native rice starch and 90% (w/w) of the respective salt solution at various concentrations. The samples were mixed for 2 minutes at 500 rpm and 25^{o}C using the magnetic stirrer. The sample was then loaded into the CC27-SS cup for rheological measurement based on the parameters and methods described in *Section 3.3*. The rice suspensions containing different mineral salts were than compared with a sample containing 10% (w/w) rice starch, 80% (w/w) liquid carom extract, and 10% (w/w) Milli-Q water, to determine whether certain mineral salts at high concentrations induced viscosity-suppressing effects.

6.2.4 Determination of the effect of phytochemicals on rice starch

The effect of phytochemicals (thymol, tannic acid, ρ-cymene, limonene, γ-terpinene, saponins, and trans-anethole) on native rice starch pasting properties was determined using rheometry. These specific phytochemicals are the major phytochemicals found in carom based on other studies (Table 3 and 4).

Two concentrations of 0.6% and 6.0% (w/w) were used for each phytochemicals. A moderate and high concentration of phytochemicals were prepared as compared to the actual amount found in carom extract to determine the extreme effects of the chemicals on starch pasting properties. Each sample (30.0 g) for rheological test consisted of 10.0% (w/w) native rice starch, 0.6% or 6.0% (w/w) of a specific phytochemical, and 84.0% or 89.4% (w/w) Milli-Q water respectively. The samples were vortexed for 3 minutes at 2500 rpm and 25^{o}C. The samples were then loaded into the CC27-SS cup for rheological measurement based on the parameters and methods described in *Section 3.3.* The rice suspensions with different phytochemicals were than compared with a sample made up of 10% (w/w) rice starch, 80% (w/w) liquid carom extract, and 10% (w/w) Milli-Q water, to determine whether any of these phytochemicals was capable of inducing viscosity-suppressing effects during starch pasting.

6.3 Results and Discussion

6.3.1 Effect of pH

The pH of rice starch, liquid carom extract, and a mixed sample of starch and carom are shown in Table 15. The addition of carom extract to rice starch reduced the pH of rice starch from 8.30 to 5.89 (Table 15), probably due to the presence of organic acids and acidic phytochemicals that have been reported in carom (Kaur & Arora, 2009).

Table 15: *pH value of different samples*

Sample (30.0 g)	pH Value
10% native rice starch and 90% Milli-Q water	8.30 (± 0.05)
Fresh liquid carom extract	5.52 (± 0.05)
10% rice starch, 10% Milli-Q water, and 80% liquid carom extract	5.89 (± 0.05)

Figure 18 and 19 show the effect of acidic and alkaline pH's on native rice starch in the absence of carom extract.

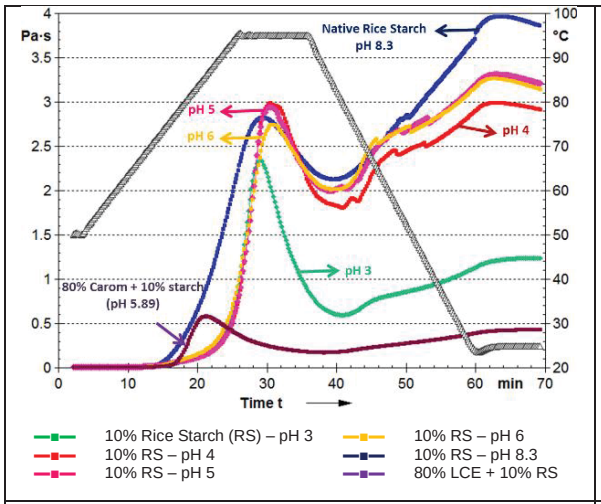


Figure 18: *The effect of acidic pH (3 – 6) on 10% (w/w) rice starch in comparison to 10% (w/w) native rice starch with 80% (w/w) Liquid Carom Extract (LCE) at pH 5.89.*

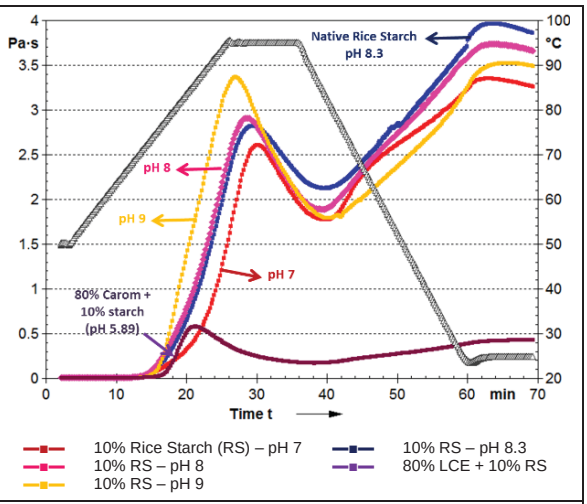


Figure 19: *The effect of neutral and alkaline pH (7 – 9) on 10% (w/w) rice starch in comparison to 10% (w/w) native rice starch with 80% (w/w) Liquid Carom Extract (LCE) at pH 5.89.*

Figure 18 shows that rice starch (without carom extract) at pH 5.00 and 6.00—close to the pH of starch-carom system—did not display a reduction in viscosity. Therefore, the suppressing effect caused by carom extract should not be associated to a change in pH.

From the screening trials of thirty-six different spice and herbal extracts, the pH of rice starch-extract mixtures ranged from 3.70 to 6.64 (Table 1). Figures 18 and 19 showed that rice starch between pH 4.00 and 7.00 did not display a significant viscosity-suppressing or enhancing effect. Only pH 3.00 showed a more pronounced decrease in viscosity but not even close to those uniquely observed in the starch-spice/herb systems. Therefore, pH was not a plausible cause for this effect or interaction.

The fact that rice starch at pH 3.00 had a slight decrease in the peak viscosity and significant decrease in the final viscosity as compared to pH 8.30 (native) can be further explained. At low pH (acidic condition), starch granules may become fragile and break down rapidly with shear and heat (H.-H. Wang et al., 2000). The rice starch systems at pH 4.00, 5.00, and 6.00 had no noteworthy changes in the peak viscosity except for a slight decrease in the final viscosity. The presence of a lower concentration of acid could still lead to a lower degree of acid hydrolysis of the amylose molecules, that results in a weaker network formation and lower final viscosity during the cooling process of the pasting curve (H.-H. Wang et al., 2000).

Regarding starch at alkaline pH as shown in Figure 19, the rice starch system at pH 7 had a slight decrease in the peak and final viscosities whereas rice starch at pH 9 had an increase in the peak viscosity and a decrease in the final viscosity. In addition, the onset of gelatinisation occurred earlier at pH 9. This could be attributed to the strengthening of bonding forces within the granules in the presence of Na^+ or OH^- ions (Bhattacharya & Corke, 1996). In addition, the OH^- may associate at specific sites in the starch and create a large hydration sphere that results in higher viscosity. However, the alkaline pH would not be able to sustain granular integrity for long, thus resulting in the rapid breakdown of the starch granules (Bhattacharya & Corke, 1996).

6.3.2 Effect of mineral salts

Carom extract contains potassium, calcium, magnesium, phosphorous, sodium, and a small quantity of iron (Table 14). These mineral salts are known to independently influence starch pasting properties based on the ion type and concentration

(Biliaderis, 2009). However, there is little literature available on the effect of different mineral salts on rice starch. Figures 20-25 show the effect of different salts and varying salt concentrations on rice starch pasting properties.

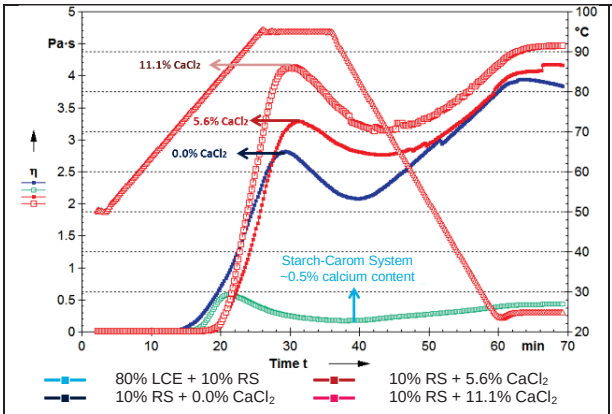


Figure 20: *The effect of 0.0% (0M), 5.6% (0.5M) and 11.1% (1.0M) calcium chloride on 10% (w/w) rice starch (RS).*

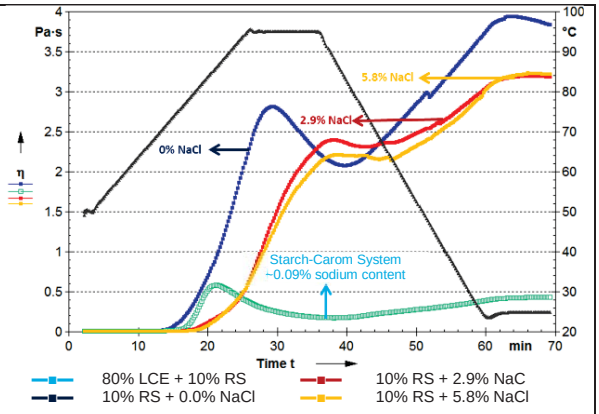


Figure 21: *The effect of 0.0% (0M), 2.9% (0.5M) and 5.8% (1.0M) sodium chloride on 10% (w/w) rice starch (RS).*

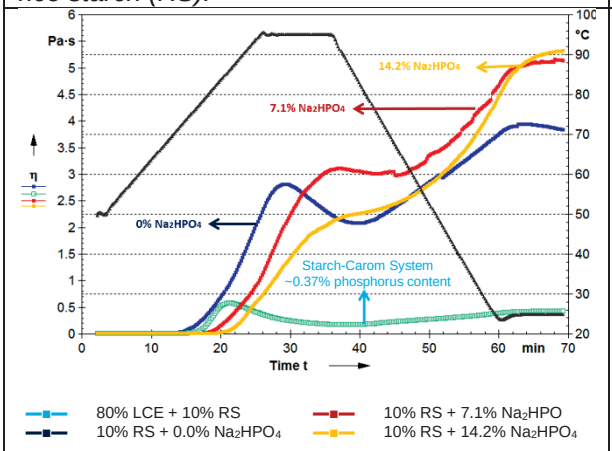


Figure 22: *The effect of 0.0% (0M), 7.1% (0.5M) and 14.2% (1.0M) sodium phosphate on 10% (w/w) rice starch (RS).*

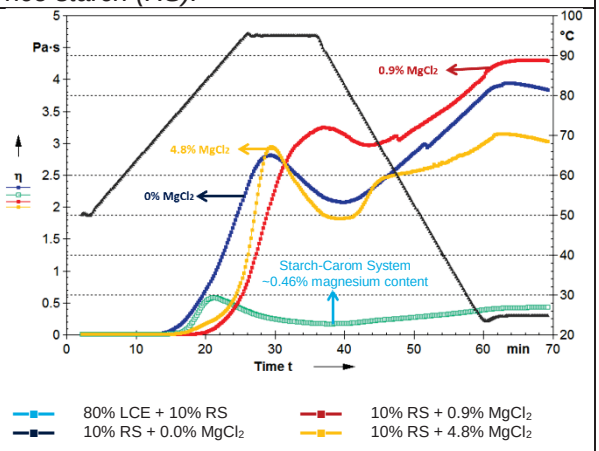


Figure 23: *The effect of 0.0% (0M), 0.9% (0.1M) and 4.8% (0.5M) magnesium chloride on 10% (w/w) rice starch (RS).*

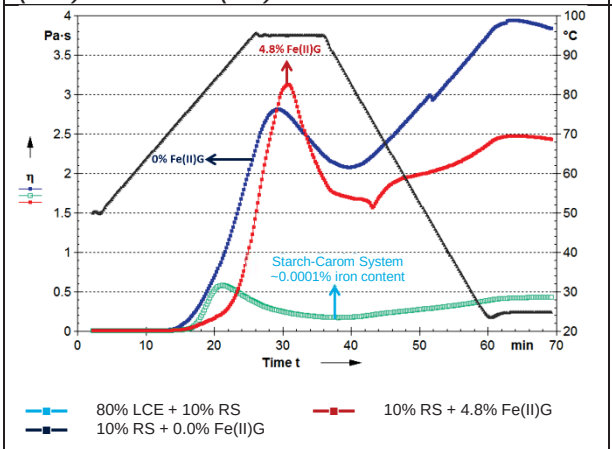


Figure 24: *The effect of 0.0% (0M) and 4.8% (0.1M) ferrous gluconate on 10% (w/w) rice starch (RS).*

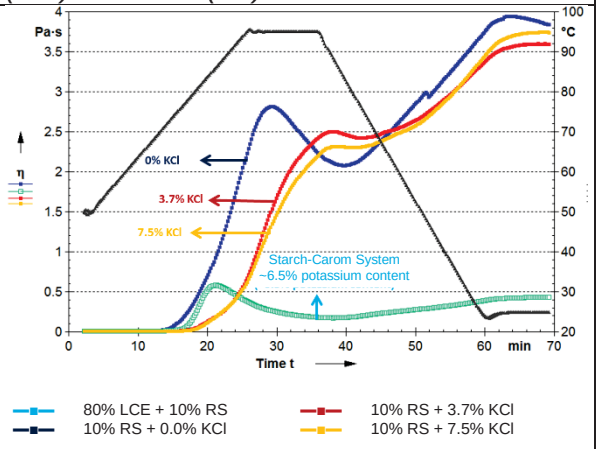


Figure 25: *The effect of 0.0% (0M), 3.7% (0.5M) and 7.5% (1.0M) potassium chloride on 10% (w/w) rice starch (RS).*

Figures 20 – 25 show that none of the mineral salts at two different concentrations displayed viscosity-suppressing effects, unlike those uniquely observed in starch-carom system. Calcium chloride (0.5M and 1.0M), sodium phosphate (0.5M and 0.1M) and magnesium chloride (0.5M) increased the peak viscosity of rice starch, while sodium chloride (0.5M and 1.0M) and sodium phosphate (1.0M) modestly decreased the peak viscosity. Salts such as sodium phosphate (0.5M and 1.0M), magnesium chloride (0.1M), and ferrous gluconate (0.1M) increased the final viscosity of rice starch, while calcium chloride (0.5M and 1.0M), magnesium chloride (0.5M), and potassium chloride (0.5M and 1.0M) decreased the final viscosity.

There are various mechanisms proposed on the effect of salts but also several reported contradicting results (Biliaderis, 2009). For example, in this study calcium chloride resulted in increased peak viscosity of rice starch. This is in agreement with the study reported by Moore et al. (1984) for maize starch, but not so in the case of potato and sweet potato starches (Jyothi et al., 2005; Moore et al., 1984).

The common understanding of the interaction is that anions (e.g. chloride) generally have a greater effect on starch pasting than cations (e.g. sodium, iron and calcium) (Biliaderis, 2009; W. Wang et al., 2015). Ions that are larger in diameter (e.g. chloride) and possess weaker electric field intensity will tend to break the links between the molecules and increase their water solubility ("salting in"). However, ions with smaller diameter (e.g. potassium) and stronger electric field intensity or polyvalent ions will tend to protect the links between the molecules and decrease the solubility of molecules ("salting out"). The “salting out” ions are able to decrease the solubility, swelling power, transparency, and particle size of starch significantly, while the “salting in” ions will increase these properties. The “salting out” ions will also increase the gelatinisation temperature, while “salting out” ions decrease it (W. Wang et al., 2015).

6.3.3 Effect of phytochemicals

Studies have shown that the thymol, ρ-cymene, S-limonene, γ-terpinene, saponins, and tannic acid are some of major compounds in carom (Table 3 and 4). The low water-solubility properties of terpenoids compounds suggests that the amount of

thymol, ρ-cymene, S-limonene and γ-terpinene maybe present in low concentration in the aqueous extract of carom (Table 5). However, an plausible explanation for the presence of these compounds in the aqueous phase could be due to the binding of these fat-soluble phytochemicals to protein present in the aqueous extract (I. Ahmad, Aqil, & Owais, 2006). Several studies have suggested that the aqueous extract of carom may have reasonable amount of thymol (fat-soluble terpenoids) to induce antimicrobial and other biological effects in *in vitro* and *in vivo* models (*refer to Section 2.3.4*).

The aqueous extract of carom may contain higher amount of water-soluble phenolic compounds (such as flavonoids and tannins) and amphipathic terpenoids (such as saponins) (Table 3). The list of phenolic compounds are very extensive and the predominant compound were not identified in this study. However, the total phenolic compounds was determined to be 21.9 mg GAE/g or 2.19% (w/w) of freeze-dried carom extract.

The effects of thymol, ρ-cymene, S-limonene, γ-terpinene, saponins, and tannic acid on rice starch pasting properties were examined in this study as shown in Figures 26 to 31.

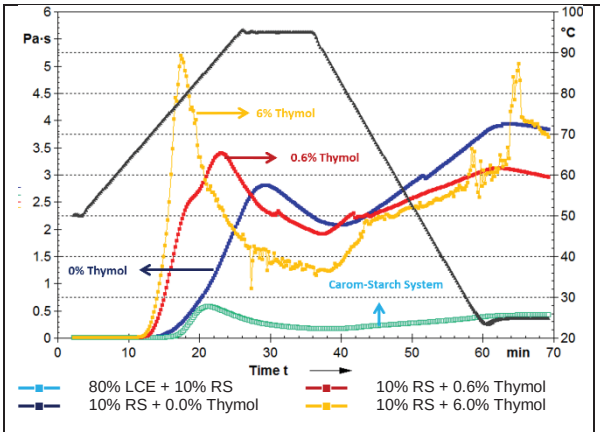


Figure 26: *The effect of 0.6% and 6.0% (w/w) thymol on 10% (w/w) rice starch (RS).*

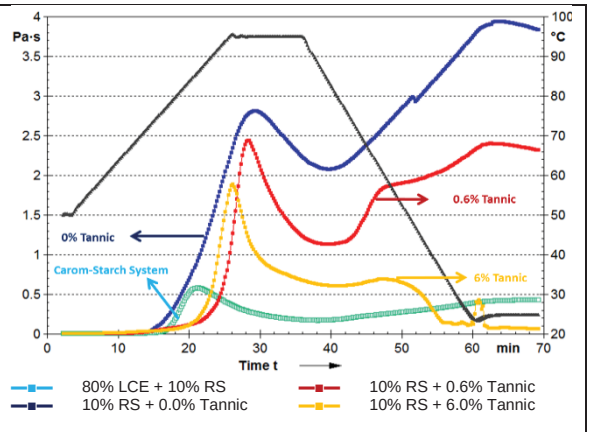


Figure 27: *The effect of 0.6% and 6.0% (w/w) tannic acid on 10% (w/w) rice starch (RS).*

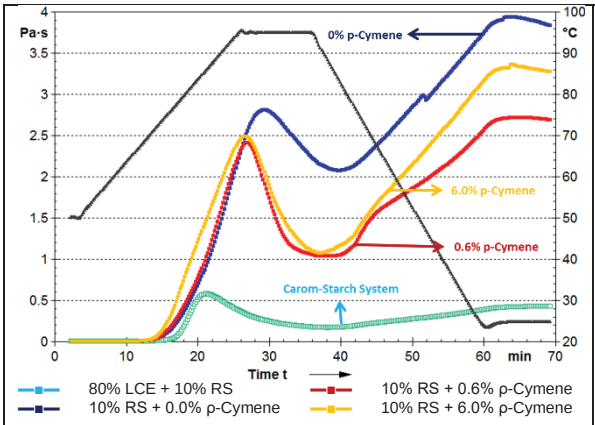


Figure 28: *The effect of 0.6% and 6.0% (w/w) p-cymene on 10% (w/w) rice starch (RS).*

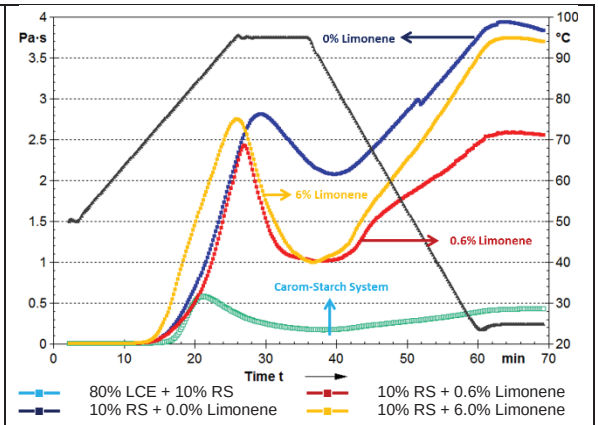


Figure 29: *The effect of 0.6% and 6.0% (w/w) S-limonene on 10% (w/w) rice starch (RS).*

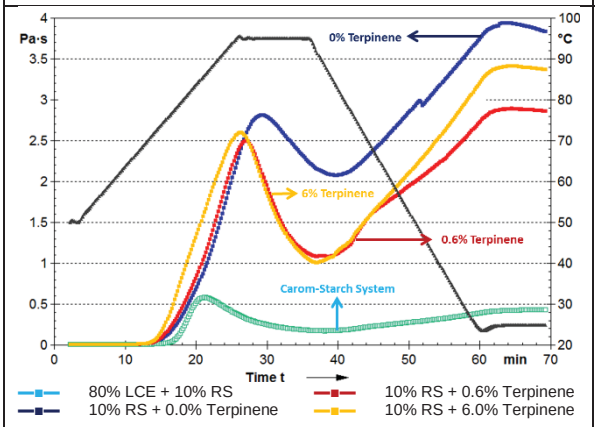


Figure 30: *The effect of 0.6% and 6.0% (w/w) γ-terpinene on 10% (w/w) rice starch (RS).*

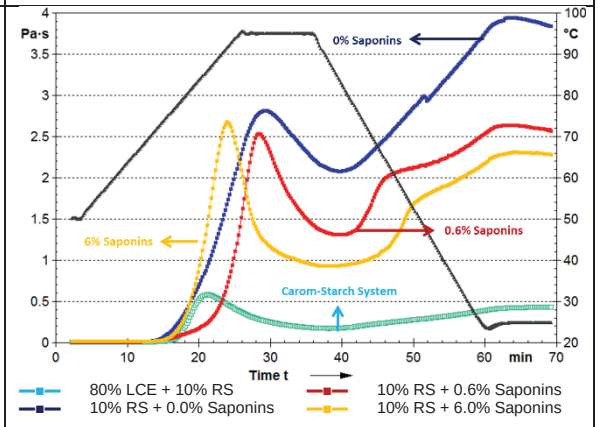


Figure 31: *The effect of 0.6% and 6.0% (w/w) saponins on 10% (w/w) rice starch (RS).*

Note: Each sample consisted of 10.0% (w/w) native rice starch, 0.6% or 6.0% (w/w) of a specific phytochemical, and 84.0% or 89.4% (w/w) Milli-Q water respectively.

It can be deduced from the graphs that thymol, tannic acid, ρ-cymene, S-limonene, γ-terpinene, and saponins within the concentration range of 0.6 to 6% w/w did not result in drastic viscosity-suppressing effect of carom extract. The concentration range of these compounds are considered wide enough to include the amount present in the carom extract. Although the exact phytochemicals and quantity in liquid carom extract were not determined in this study, the results based on the few key phytochemicals tested showed that they were not involved in the viscosity-suppressing effect of starch during pasting.

To date, there are little data on the effect of these phytochemicals on rice starch. Thymol, which is identified as the major phytochemical in carom, increased the peak and final viscosities of rice starch (Figure 26). Studies have suggested that thymol and other monoterpenoids may form “inclusion complex” with amylose helixes

through the hydrophobic bonding and/or polar interaction that involves hydrogen bonding with the hydroxyl groups of starch (Conde-Petit et al., 2006; Tietz et al., 2008a, 2008b). These complex formation could possibly cause this viscosity increment (Conde-Petit et al., 2006). However, the position of thymol in the helix and its effect on starch pasting properties are still poorly understood (Conde-Petit et al., 2006). All other phytochemicals caused a slight decrease in the peak viscosity and varying degree of decrement in the final viscosity of rice starch (Figures 26-31). Some authors suggest that these effects could be due to pH changes or the unique structural features of the phytochemicals in inducing these changes in the pasting properties (Fan Zhu, 2015; F. Zhu et al., 2008).

6.4 Conclusion

It was conclusive that the viscosity-suppressing effect caused by carom extract on rice starch was not due to the pH, presence of certain mineral salts (such as calcium chloride, potassium chloride, sodium chloride, magnesium chloride, sodium phosphate, and ferrous gluconate) and presence of certain phytochemicals (such as thymol, ρ-cymene, S-limonene, γ-terpinene, saponins, and tannic acid). Therefore, small molecular compounds present in the carom extract were not likely to be the cause of the viscosity-suppressing effect observed in starch-carom extract during starch pasting.

CHAPTER 7: CHARACTERISTICS OF CAROM POLYMER AND ITS INTERACTION WITH DIFFERENT STARCHES

7.1 Introduction

In the previous chapter, the roles of pH and small molecular compounds were studied. It was evident that the pH, certain mineral salts, and phytochemicals were not responsible for the viscosity suppressing effect of rice starch during starch pasting. In this chapter, the focus was on the polymer fractions (namely, the protein and non-starch polysaccharides). Attempts were made to determine if the proteins, a major constituent of carom extract, were responsible for the observed viscosity-suppressing effect using dialysis and protease treatment on liquid carom extract. The effects of pH and heat were also investigated.

7.2 Materials and Methods

7.2.1 Materials

Table 15 shows the starches used in the study. Apart from rice starch, obtained from Beneo GmbH, the rest were obtained from Roquette Frères (France) and Mustafa & Samsuddin Co Pte Ltd (Singapore).

Table 16: *The types of starches used and their sources.*

Starch Type	Product Name	Obtained From
Native Rice Starch	Beneo Remy ODR6	Beneo GmbH
Waxy Rice Starch	Beneo Remyline AX-DR	
Native Maize Starch	Roquette S7489	Roquette Frères
Waxy Maize Starch	Roquette N200	
Native Wheat Starch	Roquette E2615	
Native Potato Starch	Roquette VNW73	
Native Pea Starch	Roquette N735	
Native Tapioca Starch	Flying Man	Mohamed Mustafa & Samsuddin Co Pte Ltd (Singapore)
Glutinous Rice Flour	Pagoda	
Sweet Potato Flour		

7.2.2 Dialysis (dialysed carom extract)

The protein and non-starch polysaccharides (soluble fibre) in carom extract were purified using the dialysis method outlined by Scopes (1994). Samples consisting of 50.0 g of liquid carom extracts were placed in a 12,000 Da MWCO semi-permeable cellulose dialysis tubes (Spectra/Por® 2, Spectrum Inc, USA). The dialysis tubes were then placed in 5 L of Milli-Q water for 48 hours, 72 hours, and 1 week at 4^{o}C respectively. The Milli-Q water was discarded and renewed 4 times per day. The dialysed carom extract was weighed and then filtered using 0.22 µm filter membrane. The weight of the carom extract remained largely similar after dialysis, which suggested that the polymer concentration would have remained the same after the dialysis process. Any minor differences in the weight was compensated with the addition of Milli-Q water to the dialysed extract before filtration. The effect of the different dialysed extract on rice starch pasting properties was studied using the rheometer. Each sample (30.0 g) consisted of 10.0% (w/w) native rice starch, 80% (w/w) dialysed extract, and 10% (w/w) Milli-Q water. The samples were mixed for 2 minutes at 500 rpm and 25^{o}C using the magnetic stirrer. The samples were then loaded into the CC27-SS cup for rheological measurement based on the parameters and methods described in *Section 3.3*.

7.2.3 Protein hydrolysis (protease-treated carom extract)

Samples consisting of 50.0 g of liquid carom extracts were treated with 0.1% (w/w) Pronase E proteases (P5147, Protease Type XIV from *Streptomyces griseus,* Sigma-Aldrich Corporation, USA) and 0.02% (w/w) sodium azide for 0 hour, 1 hour, and 24 hours at pH 5.52 and 37.0^{o}C ± 0.5^{o}C in a water bath. Pronase E proteases are composed of five serine-type proteases, two zinc endopeptidases, two zinc leucine aminopeptidases, and one zinc carboxypeptidase.

After the treatment, the samples were filtered using 0.22 µm filter membrane to remove any form of precipitation or aggregation. The extent to which protein hydrolysis changed the molar mass of the polymer fraction after enzymatic treatment (0 hour, 1 hour, and 24 hours) was analysed using size exclusion chromatography coupled with a multi-angle laser light scattering photometer (SEC-MALLS).

The SEC-MALLS procedures were based on the procedure outlined by Goh et al. (2015). The SEC-MALLS system consisted of a guard column (SB-G F6709430, Shodex OHpak, Japan), a size exclusion column (SB-806M HQ F6429105, Shodex OHpak, Japan), a high performance liquid chromatography (HPLC) system (Agilent 1200 series, Agilent Technologies, USA), an eight-angle static light scattering detector (Agilent Technologies, USA), and a differential refractive index (DRI) detector (Agilent 1200 series, Agilent Technologies, USA) connected in series.

The mobile phase used in the experiments was Milli-Q water containing 0.1 M sodium chloride and 0.02% (w/w) sodium azide (Merck Millipore Corporation, USA). The solvent was filtered using 0.025 μm membrane filter (Merck Millipore Corporation, USA) and degassed under vacuum for 1 hour at room temperature prior to use. All glass apparatus were pre-washed in 5.0% (w/w) nitric acid and rinsed thoroughly using Milli-Q water.

The carom extract samples (50 μL) after enzymatic treatment (0 hour, 1 hour, and 24 hours) were injected into the column with eluent flowing at 0.5 mL/min using a HPLC pump. The light scattering data was recorded using Astra V software (Version 5.3.4.20, Wyatt Technology Corp., CA, USA). The Astra V software was used to analyse the light scattering data using Zimm plot to derive the weight-average molar mass (M_w) of the carom polymer before and after 0.1% (w/w) protease treatment at 0 hour, 1 hour, and 24 hours.

The protease-treated liquid carom extracts (0 hour, 1 hour, and 24 hours) were also added to rice starch to evaluate its pasting properties using the rheometer. Each sample (30.0 g) consisted of 10.0% (w/w) native rice starch, 80% (w/w) protease treated extract (0 hour, 1 hour, and 24 hours), and 10% (w/w) Milli-Q water. The samples were mixed for 2 minutes at 500 rpm and 25^{o}C using the magnetic stirrer. The samples were then loaded into the CC27-SS cup for rheological measurement based on the parameters and methods described in *Section 3.3*.

7.2.4 Determination of the effect of pH on carom extract

7.2.4.1 Visual observation

The pH of 10 mL fresh liquid carom extract was adjusted to pH 2.0 - pH 9.0 by the addition of 1M HCl or 1M NaOH respectively. The final volume was then standardised to 12 ml with Milli-Q water. The carom extracts were allowed to stand at 25^{o}C for 1 hour to achieve pH equilibrium.

7.2.4.2 Isoelectric point of carom protein

The isoelectric point of the protein in carom extract was determined by electrokinetic measurements using the zetasizer (Nano ZS ZEN 3600, Malvern Instruments Ltd., Malvern, Worcestershire, UK). The protocol for zeta potential measurement was modified based on the procedure outlined by Matia-Merino et al. (2012). The zeta potential values of liquid carom extract at different pH values were measured based on an optimum dilution (using 3.3% of liquid carom extract diluted in Milli-Q water). The pH of the diluted liquid carom extract in Milli-Q water was then adjusted to pH 2.0 - pH 9.0 by the addition of 1M HCl or 1M NaOH respectively.

Approximately 1 mL of each carom sample was introduced into a disposable folded capillary zeta cell (DTS 1060, Malvern Instruments Ltd, UK). Any traces of air bubbles were removed, before inserting the stopper. The zeta potential measurements were performed at 25.0°C ± 0.1^{o}C. All measurements were carried out on two fresh liquid carom extract samples and data were reported as means.

7.2.4.3 Pasting properties using "pH-treated carom extract"

The pH of 50.0 g of liquid carom extract was adjusted to achieve pH 2 - pH 9 using either 1M HCl and NaOH. All the extracts were filtered using 0.22 µm filter membrane to remove any form of precipitation or aggregation. The pH of the samples were readjusted back to the native pH of 5.52. The effect of the pH-treated carom extract on rice starch pasting properties was determined using the rheometer. Each sample (30.0 g) consisted of 10.0% (w/w) native rice starch, 80% (w/w) pH-treated carom extract, and 10% (w/w) Milli-Q water. The samples were mixed for 2 minutes at 500

rpm and 25ºC using the magnetic stirrer. The samples were then loaded into the CC27-SS cup for rheological measurement based on the parameters and methods described in *Section 3.3*.

7.2.5 Determination of the effect of heat on carom extract

7.2.5.1 Structural characteristics of heated carom protein

The morphology and microstructure of polymers in carom extract and starch-carom gels (with and without carom) were observed using scanning electron microscopy (SEM).

A 10.0% (w/w) freeze-dried carom extract was rehydrated in Milli-Q water and dialysed (*refer to Section 7.2.2*). A portion of the dialysed carom extract was then heated at 90ºC ± 1ºC for 5 minutes in a water bath. The unheated and heat-treated carom solutions were then added with fixatives. The samples were initially fixed in modified Karnovsky's fixative [3% (w/w) glutaraldehyde and 2% (w/w) formaldehyde in 0.1M phosphate buffer (pH 7.2)] for at least 8 hours. Each sample was centrifuged at 7000 rpm for 1 minute at 25ºC. The samples were rinsed three times in 0.1M phosphate buffer (pH 7.2), then dehydrated in a graded series of ethanol (25%, 50%, 75%, 95%, and 100%) (w/w) for 15 minutes each and a final 100% for 1 hour. Samples undergo critical point drying (Polaron E3000 series II critical point drying apparatus, Loughborough, UK) using liquid carbon dioxide and 100% ethanol as the intermediary. The dried samples were then mounted on an aluminium stub, sputter coated with approximately 100nm of gold (BAL-TEC SCD 005 sputter coater, SEM/EDX Laboratory, Germany) and viewed in a scanning electron microscope (FEI Quanta 200, FEI, USA) under high vacuum with at an accelerating voltage of 20kV. Each sample were analysed in duplicates.

7.2.5.2 Pasting properties using heat-treated carom extract

Samples consisting of 50.0 g of liquid carom extracts were treated to 20, 40, 60, 70, 80, 83, 85 and 100ºC ± 1 ºC respectively for 5 minutes in a water bath before cooling to 20ºC ± 1 ºC in the water bath. The extracts were filtered using 0.22 µm filter

membrane to remove any form of precipitation or aggregation. Each sample (30.0 g) consisted of 10.0% (w/w) native rice starch, 80% (w/w) heat-treated carom extract, and 10% (w/w) Milli-Q water. The samples were mixed for 2 minutes at 500 rpm and 25^{o}C using the magnetic stirrer. The samples were then loaded into the CC27-SS cup for rheological measurement based on the parameters and methods described in *Section 3.3*.

7.2.6 Determination of the effect of carom extract on different starches

7.2.6.1 Rheological measurement

The effect of carom extract on different starches (Table 15) were determined using the rheometer. Each sample (30.0 g) consisted of 10.0% (w/w) specific starch, 80% (w/w) liquid carom extract, and 10% (w/w) Milli-Q water. The samples were mixed for 2 minutes at 500 rpm and 25^{o}C using the magnetic stirrer. The samples were then loaded into the CC27-SS cup for rheological measurement based on the parameters and methods described in *Section 3.3*.

7.2.6.2 Hot-Stage Optical Microscopy (HSOM) Analysis

The method is similar to those described in *Section 3.4*. The effect of carom extract on the swelling of different starches (potato, maize, wheat, pea, tapioca, and sweet potato) were studied. A starch-carom suspension (0.50 g) containing 1.5% (w/w) a specific starch, 3.5% (w/w) freeze-dried carom extract, and remaining with Milli-Q was prepared in an Eppendorf plastic tube (2 mL). The suspension was mixed thoroughly at 500 rpm for 1 minute using a vortex mixer. Approximately 200 µL of suspension was transferred onto a glass slide and studied according to procedure described in *Section 3.4*.

7.2.7 Determination of structural characteristics of starch-carom system during pasting

A sample (30.0 g) consisting of 10.0% (w/w) native rice starch, 80% (w/w) liquid carom extract, and 10% (w/w) Milli-Q water were mixed and loaded into the CC27-

SS cup to be heated and cooled based on parameters and methods described in *Section 3.3*. Approximately 1 g sample was drawn at different intervals [25^{o}C, 70^{o}C, 85^{o}C, 95^{o}C, 25^{o}C (cooling)] during the heating and cooling process. The remaining gel was refrigerated for 24 hours and 1 g of sample was drawn from the refrigerated gel after 24 hours. The samples were then analysed using SEM as described in *section 7.2.5.1*, to determine any structural changes during the pasting process.

7.3 Results and Discussion

7.3.1 Effect of dialysed carom extract

The liquid carom extract was dialysed for 48 hours, 72 hours, and 1 week *(refer to Section 7.2.2*). The purpose of dialysis was to remove small molecular compounds (e.g. minerals, sugars, acids, and phytochemicals) and retain the polymer fractions (Scopes, 1994). However, some phytochemicals such as tannins may strongly be bound to the protein molecules and may not be removed through dialysis. Figure 32 shows the effect of different dialysed extracts (0 hours, 48 hours, 72 hours, and 1 week) on starch pasting properties.

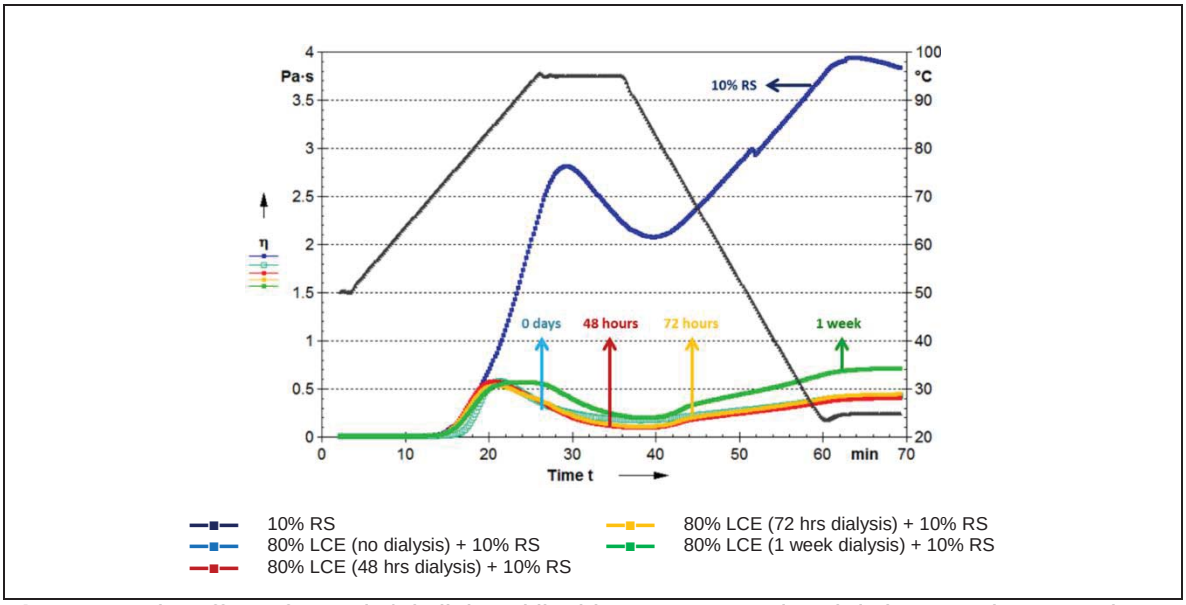


Figure 32: *The effect of 80% (w/w) dialysed liquid carom extract (LCE) (0 hour, 48 hours, 72 hours, and 1 week) on the pasting properties of 10% (w/w) rice starch (RS)*

All the dialysed carom extracts had very similar viscosity-suppressing effect on rice starch. The 1 week dialysed sample had a slight shift and increment in the viscosity, probably due to microbial growth or hydrolysis of the active polymer after prolonged storage. The results thus far (*refer to Chapter 6*) have provided clear evidence that the small molecular compounds below the molecular weight of 12, 000 Da are not responsible for the viscosity-suppressing effect. Therefore, the larger molecular species such as proteins and/or non-starch polysaccharide (Table 14) in the extract are possibly responsible for the viscosity-suppressing effect of starch during pasting.

7.3.2 Effect of protease-treated carom extract

The liquid carom extracts were treated with Pronase E (mixture of proteases) for 0 hour, 1 hour, and 24 hours (*refer to Section 7.2.3*). The protease treatment would hydrolyse the proteins in the extract and elucidate the role of protein in the viscosity-suppressing effect. Figures 33 (a-f) shows the effect of different protease-treated carom extract (0 hour, 1 hour and 24 hours) on the changes in the molecular mass distribution of the polymer in the extract and pasting properties of rice starch.

Figure 33a shows the light scattering and refractive index chromatograms of untreated or 0 hour carom extract (control) analysed by SEC-MALLS. Figure 33b shows the effects of the same extract on the pasting properties of rice starch analysed in the rheometer. Similarly, Figure 33c and 33d show the effect of 1 hour protease treatment, while Figure 33e and 33f show the effect of 24 hours treatment.

The Zimm fit method was used to fit all the light scattering data. The weight-average molecular weight (M_w) of the polymer fraction (Figure 33a) determined by the Astra software based on Rayleighe-Debyee-Gans equation was $2.08 \pm 0.10 \times 10^5$ Da. The polydispersity index (M_w/M_n) of the polymer fraction was relatively low at 1.13 indicating a fairly monodispersed fraction. The protease treatment for 1 hour and 24 hours reduced the M_w of the polymer fraction in carom extract to $9.26 \pm 0.03 \times 10^4$ Da (Figure 33c) and $2.52 \pm 0.02 \times 10^4$ Da (Figure 33e) respectively. The reduction in the M_w of the protein after 1 hour and 24 hours protease treatment (observed as a reduction in the light scattering peak) correlated to the loss of viscosity-suppressing effect in the pasting curves. Figure 33d and 33f show that the peak and final viscosities are less suppressed after protease treatment. From the results, it is reasonable to deduce that the protein fraction from carom with M_w of $\sim 2.08 \pm 0.10 \times 10^5$ Da is responsible for the viscosity-suppression effect of rice starch during pasting. Kaur et al. (2009) reported the presence of an anti-calcifying protein in carom aqueous extract with a molecular weight of $\sim 1.07 \times 10^5$ Da that was analysed using a matrix-assisted laser desorption/ionization.

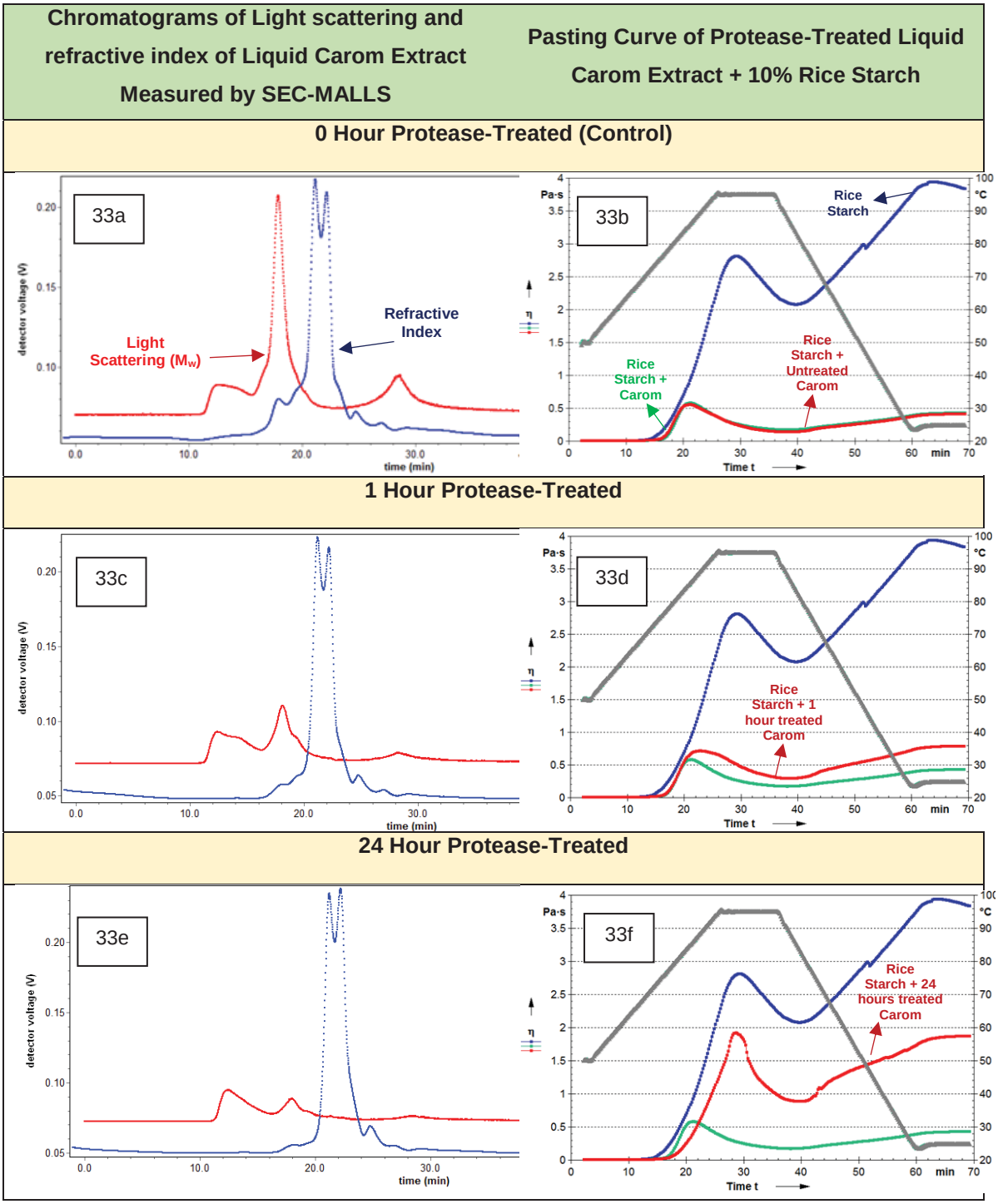


Figure 33(a-f): *The effect of protease treatment (0 hour, 1 hour, and 24 hours) on the molecular mass distribution (33a, 33c and 33e) of carom extract liquid and its corresponding changes in rice starch pasting properties (33c, 33d and 33f) respectively.*

Note:
The (—) represents the molecular mass distribution based on the light scattering peak and (—) represents refractive index in Figures 33a, 33c and 33e.

The (—) represents pasting curve of native rice starch, (—) represents pasting curve of rice starch and carom extract (without 0.1% protease), and (—) represents pasting curve of rice starch and carom extract (with 0.1% protease) in Figures 33b, 33d and 33f.

7.3.3 Structural characteristics of carom protein

Scanning electron microscopy (SEM) is typically used in the examination of polymer microstructures found in food (James, 2014). Figure 34 shows the SEM images of the polymer found in dialysed (24 hours) 10% (w/w) carom extract.

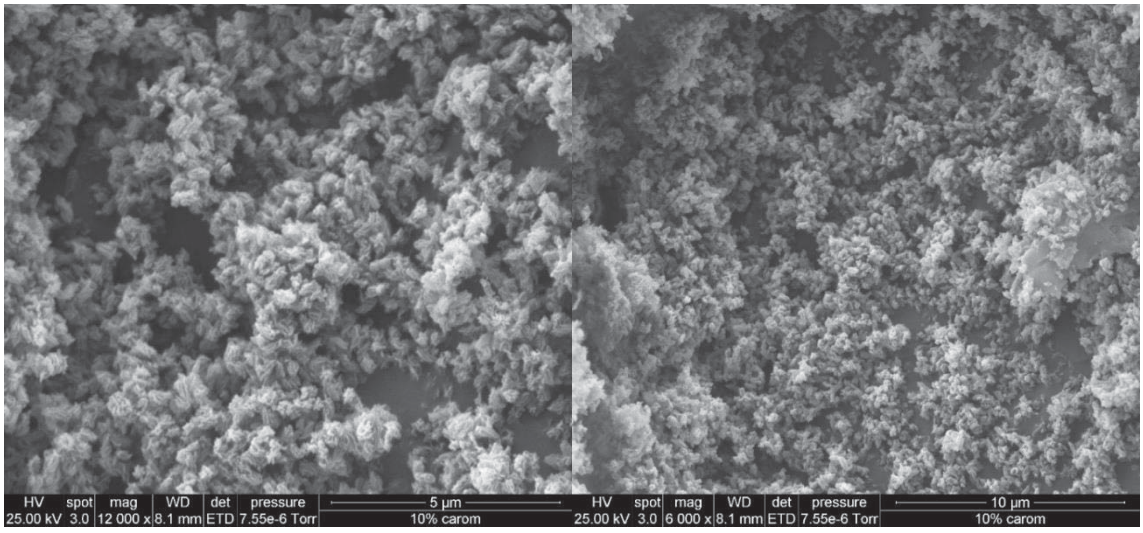


Figure 34: *Scanning electron microscope images (5µm and 10µm) of polymers identified in dialysed (24 hours) 10% (w/w) carom extract solution.*

Figure 34 shows a unique aggregated raisin-like cluster appearance that is uncommon in typical proteins or polysaccharides SEM images. The appearance of these proteins resembles structures of whole milk coagulated using *Streptococcus thermophilus* during cheese production (Figure 35a) and soy cheese spread coagulated using lactic acid bacteria and papain (Figure 35b). There is no literature available on the protein structures isolated from the fruits of Apiaceae family of plants for comparison.

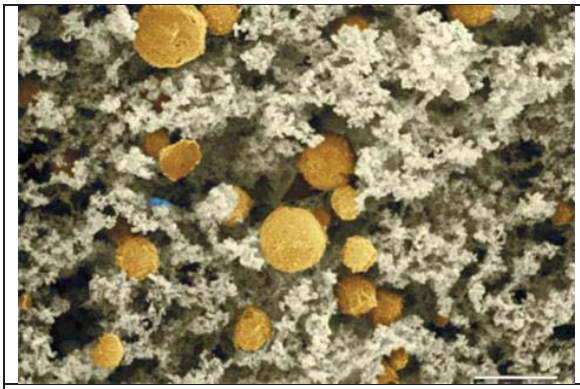


Figure 35a: *SEM image of coagulated whole milk. The matrix was casein "sponge" (white) with milkfat globules (yellow). The bottom-right bar represents 5µm. Source: (Kaláb, 2010)*

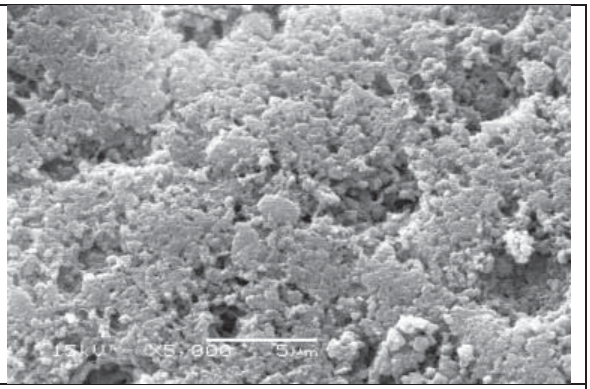


Figure 35b: *SEM image of soy cheese spread sample SCS-B, which was made by lactic acid bacteria fermentation method and modified by papain. The bottom-left bar represents 5µm. Source: (Q. Li, Xia, Zhou, & Xie, 2013)*

7.3.4 Effect of pH on carom extract

7.3.4.1 Isoelectric point of carom protein

The behaviour and characteristics of proteins are easily influenced by pH (Hall, 1996). The isoelectric point (p*I*) is an important characteristic of proteins. The p*I* of the protein in the diluted carom extract was determined using electrokinetic measurements (*refer to Section 7.2.4.2*). Figure 36 shows the zeta potential value of carom extract at different pH´s.

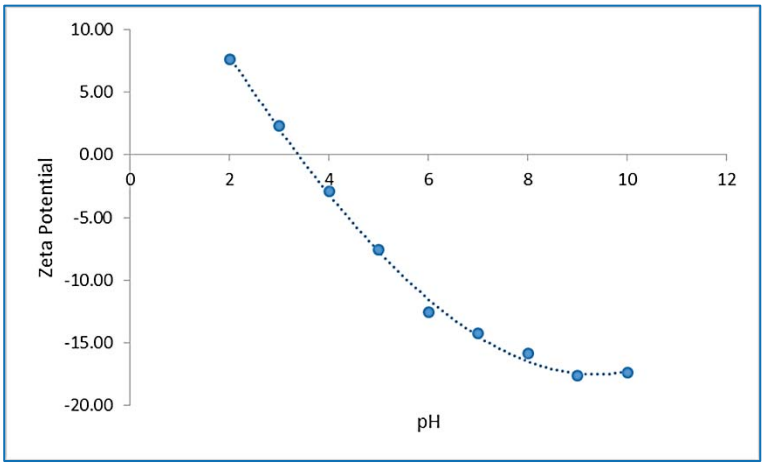


Figure 36: *Zeta potential (mV) measurement at 20 °C of diluted liquid carom extract samples with pH adjusted between 2 and 10.*

Figure 36 shows that the isoelectric point (p*I*) of carom protein was ~3.5. At pH 3.5, the protein in carom extract would have equal numbers of positive and negative charges on its surface that result in a net charge of zero. As expected, based on the results in Figure 36, carom protein had minimal solubility and aggregated at pH 3 to 4 (Figure 37). At pH values below and above isoelectric point of pH 3.5, carom protein would carry a net positive or net negative charge respectively. In general, the presence of surface charges is important since it will result in intermolecular repulsion promoting solubilisation of proteins (Butt, Graf, & Kappl, 2004; Culbertson, 2005).

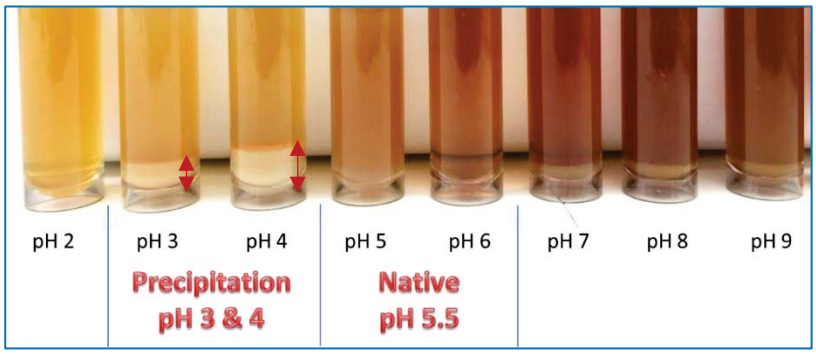


Figure 37: *Visual appearance of liquid carom extract with changing pH from pH 2.0 (left) to pH 9 (right).*

Most proteins have p*I* of 4 – 9 due to the p*I* of individual amino acids that makes up the protein molecule. However, only certain proteins such as pepsin, glycosylated α-acid glycoprotein, acetylated bovine serum albumin, and phosphorylated phosvitin have isoelectric point below 4 (J. Wang & Dupont, 2012). The net charge of a protein is dependent on the relative number of acidic and basic amino acids residues (S Damodaran et al., 2008). Aspartic acid and glutamic acid are the only amino acids with p*I* lower than 4. Therefore, the low isoelectric point of carom protein suggest that the sum of aspartic acid (p*I*=2.77) and glutamic acid (p*I*=3.22) residues in carom protein may possibly be greater than the sum of arginine (p*I*=10.76), histidine (p*I*=7.59) and lysine (p*I*=9.74) residues (S Damodaran et al., 2008). Therefore, the acidic nature of carom protein exhibited a minimum solubility at low pH (3 - 4) and maximum solubility at alkaline pH.

Kaur et al., (2009) reported that the anti-calcifying protein isolated from carom had a isoelectric point of 6.2 (T. Kaur et al., 2009). In addition, amino acid analysis showed abundant presence of acidic amino acids such as aspartic acid and glutamic acid. The protein isolated in their study had different molecular weight and isoelectric point compared to the present study, probably due to the difference in the extraction and purification method and the origin of carom.

7.3.4.2 Effect of pH-treated carom extract on starch pasting

The liquid carom extracts were pH treated (2 to 9); then filtered to remove precipitation; and readjusted back to the native pH of 5.52 (*refer to Section 7.2.4.3*). These pH treated samples were then studied for their effect on starch pasting

properties. Figure 38 and 39 show the effect of these pH-precipitated carom extract on rice starch pasting properties. All the samples (rice starch with carom extract) shown in Figures 38 and 39 had a final pH of 5.89. The carom extracts pre-treated at pH 2 and 3 lost their viscosity suppressing effect completely. There was a moderate suppressing effect observed for carom extract pre-treated at pH 3.5 and 9, while pre-treatment at pH 4, 5, 6, 7, and 8 showed relatively good viscosity suppressing effect. The highest viscosity suppression was observed for the sample at native pH of 5.5.

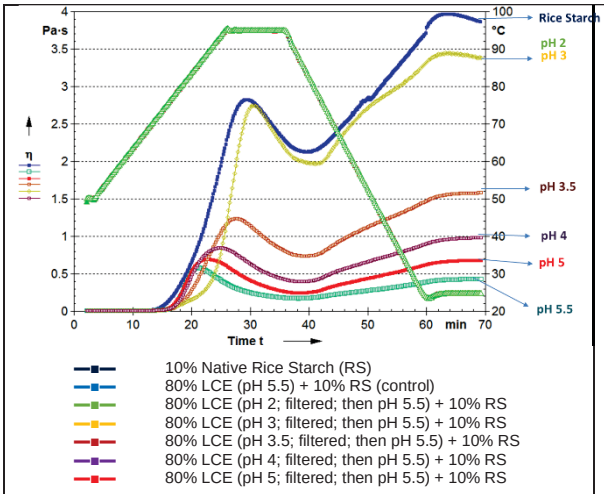


Figure 38: *The effect of pH-treated (pH 2-5) liquid carom extract (LCE) on pasting properties of rice starch (RS).*

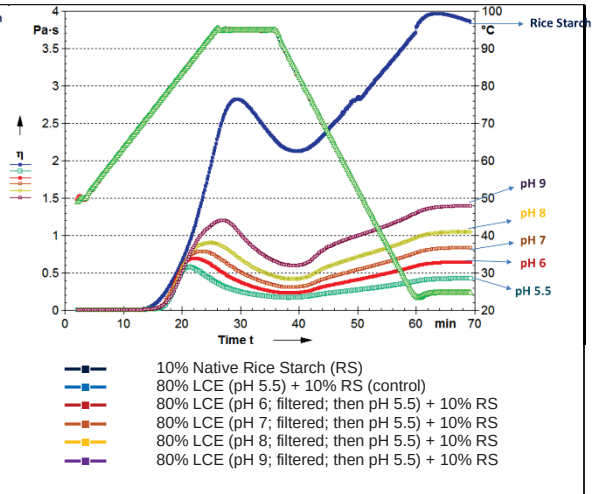


Figure 39: *The effect of pH-treated (pH 6-9) liquid carom extract (LCE) on pasting properties of rice starch (RS).*

Although the *pI* of carom protein was found to be at pH 3.5 (Figure 36) and the highest amount of precipitates were observed at pH 4 (Figure 37), the actual protein fraction responsible for suppressing the viscosity of starch during gelatinisation appeared to be denatured at approximately pH 3 (Figure 38). Most of the proteins that aggregated/precipitated and were filtered out at pH 3.5 and 4 were not the major proteins that contributed to the viscosity-suppressing effect, as only a moderate viscosity suppressing effect was observed in the rice starch pasting curve. At extreme pH conditions (e.g. pH 2 and 9), strong intramolecular electrostatic repulsion caused by a high net charge can result in swelling and unfolding of the protein molecules (S Damodaran et al., 2008) . At extremely low pH (e.g pH 2), proteins can also undergo acid hydrolysis that may result in the loss of the suppression effect or interaction with starch (S Damodaran et al., 2008).

7.3.5 Effect of heat on carom protein

7.3.5.1 Effect of heat treatment on the structure of carom extract

Figure 40 shows the SEM micrographs of carom extract with and without heat treatment (*refer to Section 7.2.5.1*). The micrographs show that heating resulted in large aggregates that could be a result of heat-induced denaturation and aggregation of the protein molecules.

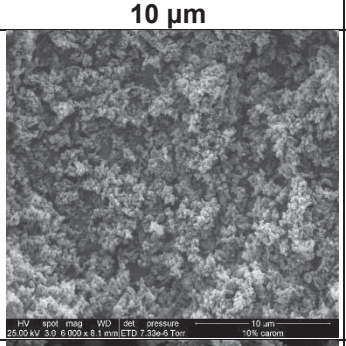
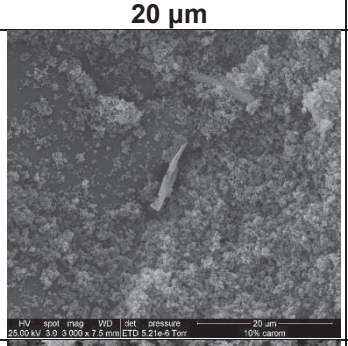
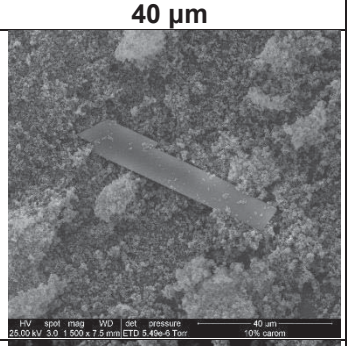
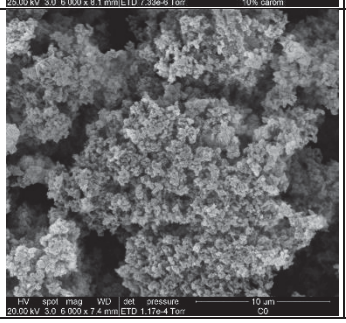
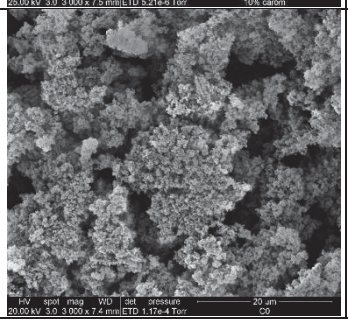
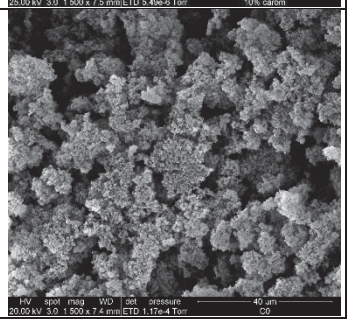
Size	10 µm	20 µm	40 µm
Before Heating			
After Heating (90°C for 5 mins)			

Figure 40: *SEM images (10, 20 and 40µm) of dialysed (24 hours) 10% (w/w) freeze-dried carom extract solution with and without heat treatment (90°C for 5 mins).*

7.3.5.2 Effect of heat-treated carom extract on starch pasting

Liquid carom extracts were heat treated at different temperature of 20, 40, 60, 70, 80, 83, 85 and 100°C for 5 minutes and filtered (*refer to Section 7.2.5.2*). Figures 41 and 42 shows the effect of heat pre-treated carom extracts on rice starch pasting.

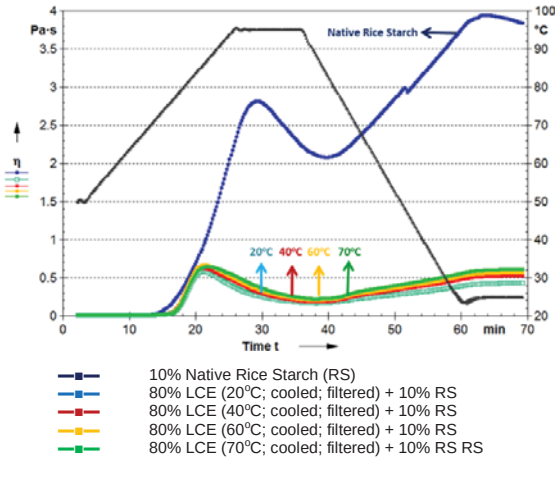


Figure 41: *The effect of preheated (20, 40, 60, 70°C) liquid carom extract (LCE) on pasting properties of rice starch (RS).*

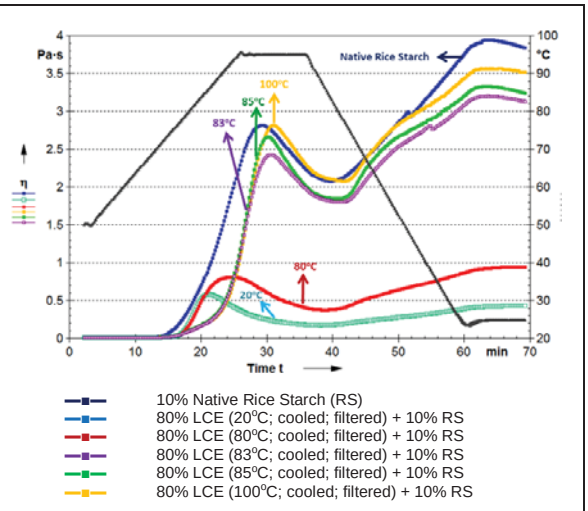


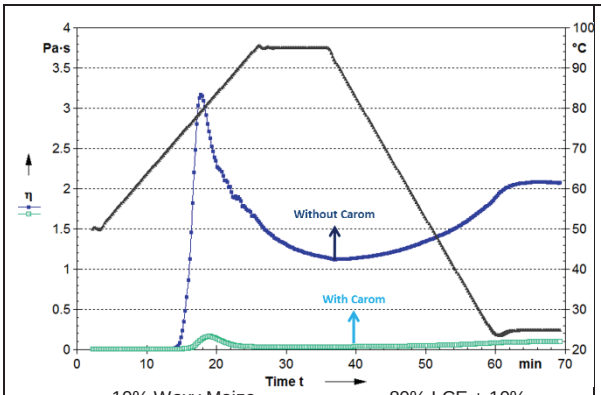
Figure 42: *The effect of heat preheated (20, 80, 83, 85, 100°C) liquid carom extract (LCE) on pasting properties of rice starch (RS).*

Heating carom extracts at 20°C, 40°C, 60°C and 70°C and subsequently added to starch showed similar viscosity suppression of starch during pasting (Figure 41). However, the extract heated at 80°C partially lost its suppressing effect. The effect was completely lost for extracts heated to 83°C, 85°C and 100°C (Figure 42). Therefore, the protein in carom was not heat stable above 70°C. The protein was likely to have partially denatured at 80°C and was completely denatured at above 83°C. The thermal denaturation temperature of carom protein was not specifically quantified in this study. Proteins such as α-lactalbumin, cytochrome C, and β-lactoglobulin denature at 83°C, which appears to be similar to the thermal denaturation point of protein in carom extract.

7.3.6 Effect of carom extract on different starches

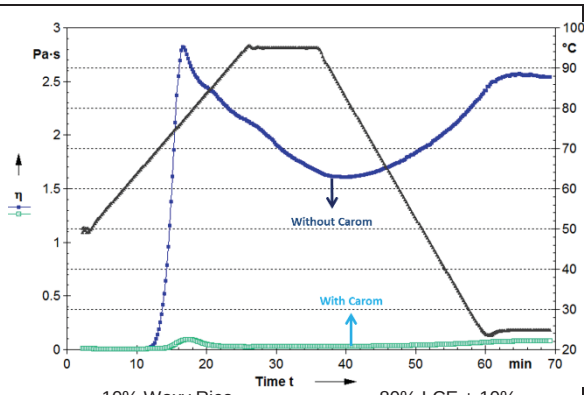
7.3.6.1 Rheological properties

Figures 43 (a-j) shows the effect of carom extract on the pasting properties of different starches with varying amylose content.



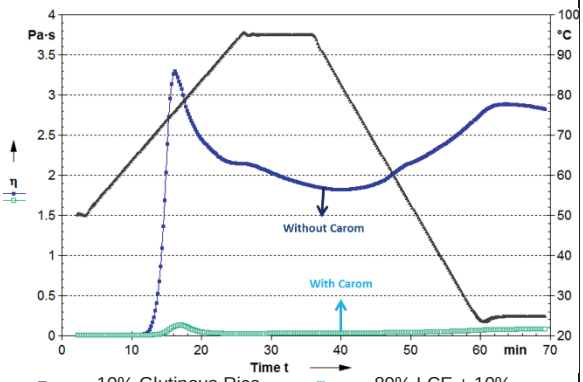
10% Waxy Maize Starch — 80% LCE + 10% Waxy Maize Starch

43a: ~ 1% Amylose (Cereal)



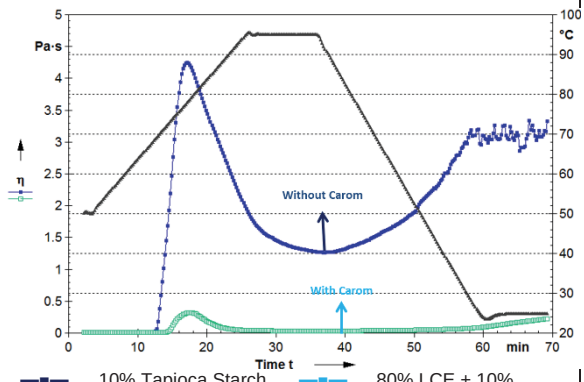
10% Waxy Rice Starch — 80% LCE + 10% Waxy Rice Starch

43b : ~ 1% Amylose (Cereal)



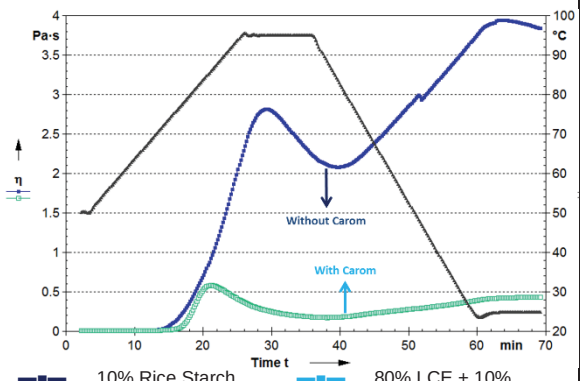
10% Glutinous Rice Flour — 80% LCE + 10% Glutinous Rice Flour

43c : ~ 1-4% Amylose (Cereal)



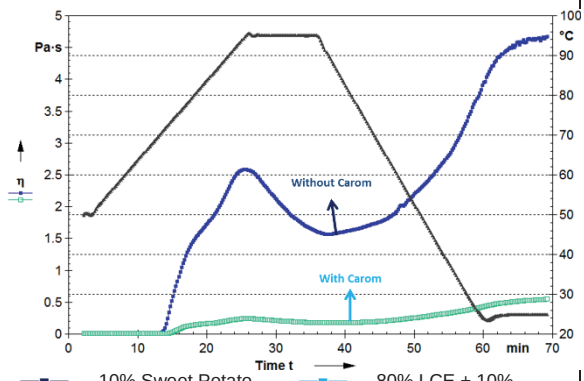
10% Tapioca Starch — 80% LCE + 10% Tapioca Starch

43d : ~ 17% Amylose (Root tuber)



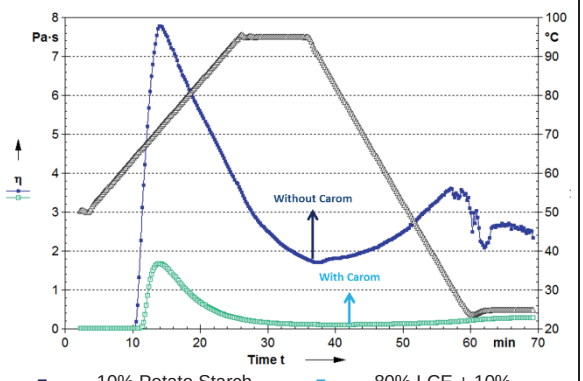
10% Rice Starch — 80% LCE + 10% Rice Starch

43e : ~17% Amylose (Cereal)



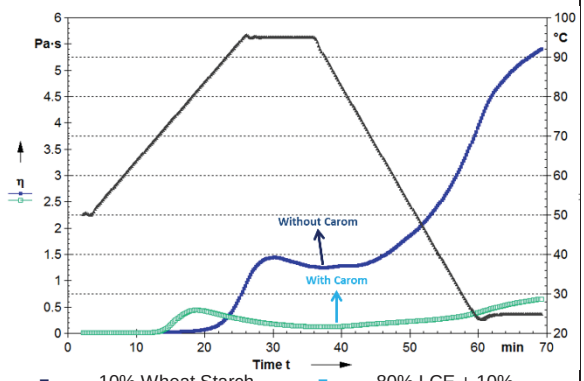
10% Sweet Potato Starch — 80% LCE + 10% Sweet Potato Starch

43f : ~ 21-24% Amylose (Root tuber)



10% Potato Starch — 80% LCE + 10% Potato Starch

43g : ~ 21-22% Amylose (Stem tuber)



10% Wheat Starch — 80% LCE + 10% Wheat Starch

43h : ~ 25-28% Amylose (Cereal)

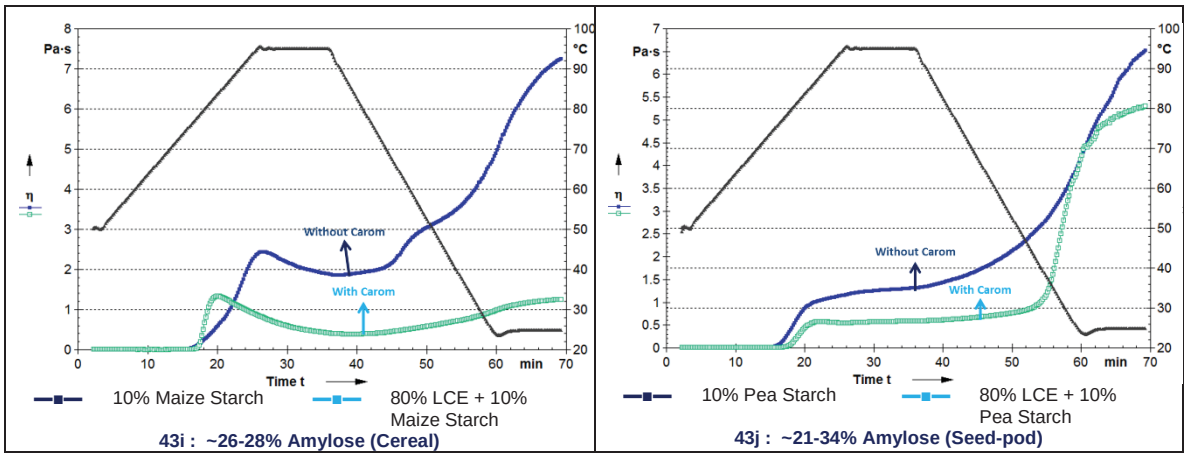


Figure 43(a-j): *The effect of liquid carom extract (LCE) on different starches (potato, tapioca, glutinous rice, waxy maize, waxy rice, rice, sweet potato, maize, wheat and pea) with varying amylose content.*

The following starches (without carom extract) had high peak viscosities in descending order: potato, tapioca, glutinous rice, waxy maize, waxy rice, rice, sweet potato, maize, wheat, and pea. While the following starches (without carom extract) had high final viscosities in descending order: maize, pea, wheat, sweet potato, rice, tapioca, glutinous rice, waxy rice, potato, and waxy maize.

Tuber and waxy starches, which are generally high in amylopectin, showed high peak viscosities, except for sweet potato starch. While, cereal starches and pea starch, which are generally high in amylose content, had lower peak viscosities. Cereal, pea, and sweet potato starches had high final viscosities, while the other tuber and waxy starches had lower final viscosities. All this follows the known trends for the various types of starches studied here based on their structure and chemical composition (A. Brown, 2008; Collado & Corke, 2003; S. Damodaran et al., 2008).

Swelling during gelatinisation is a property of amylopectin that results in an increase in the peak viscosity (Fredriksson et al., 1998; Tester & Morrison, 1990). Amylopectin is also less prone to gelation and retrogradation during the cooling process due to the branched structure (A. Brown, 2008; Collado & Corke, 2003; S. Damodaran et al., 2008). Therefore, starches with high amylopectin content (waxy and tubers) can be expected to have higher peak viscosities and lower final viscosities as compared to high amylose starches (cereals and pea).

Amylose molecules are linear chains and tend to associate strongly via extensive intermolecular hydrogen bonds making them less prone to hydration (Fredriksson et al., 1998; Tester & Morrison, 1990). During the cooling process after gelatinization, the amylose molecules re-associate to form a three dimensional network that traps water and increases the final viscosity as the gel forms (A. Brown, 2008; Collado & Corke, 2003; S. Damodaran et al., 2008). Therefore, high amylose starches (cereals and pea) usually have lower peak viscosity and higher final viscosity.

Cereal starches also contain a high percentage of lipids (~0.8–0.9%) as compared to tubers (~0.1%), which represses the swelling and solubilisation of the starch granules that results in a lower peak viscosity than other starches (Collado & Corke, 2003; W. Wang et al., 2015). The lipids also increase the pasting temperatures and reduce the water-binding ability of these starches.

Figure 44 is a replot of Figures 43 (a-j) that shows the viscosity difference in the peak and final viscosities of starch and starch-carom system for different starches

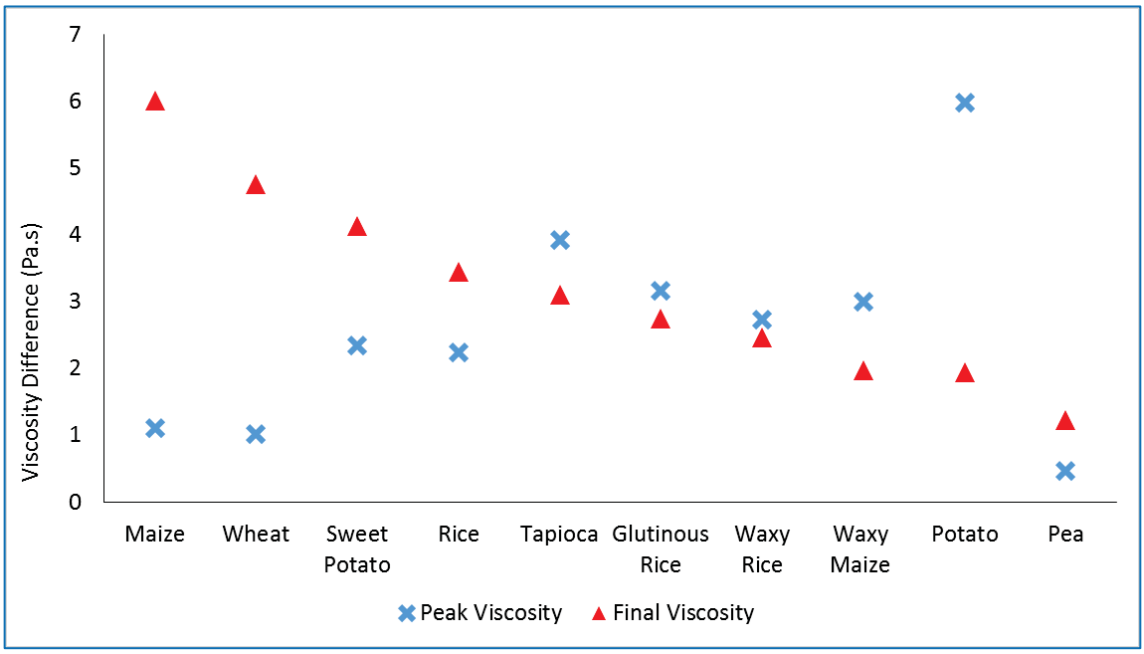


Figure 44: *The viscosity difference in the peak and final viscosities of starch and starch-carom system for different starches (potato, tapioca, glutinous rice, waxy maize, waxy rice, rice, sweet potato, maize, wheat, and pea).*

Based on Figures 43 and 44, carom suppressed the peak and final viscosities of all the starches that were studied. Carom extract had minimal effect on pea starch as both the peak and final viscosities were not affected drastically. Generally, high

amylopectin starches such as potato, tapioca, and waxy starches had a drastic reduction in peak viscosity in the presence of carom extract. On the other hand, high amylose starches such as maize, wheat, and rice had a drastic reduction in the final viscosity in the presence of carom extract. It was evident from Figure 44 that carom extract was able to suppress the swelling characteristics of high amylopectin starches by reducing the peak viscosity. It was also able to hinder the gel network formation of high amylose starches and reducing the final viscosity of the starches. The fact that viscosity-suppressing effects were observed in waxy starches (very low in amylose content) implied that carom protein could potentially influence both amylose and amylopectin independently.

In addition, studies have shown that starch granule surfaces consist of granule-associated proteins and phospholipids, which charges the granules (Baldwin, 2001; Ryan & Brewer, 2005b, 2006). This could lead to a possible electrostatic interaction with carom proteins resulting in a peak and final viscosity suppression. Hydrophobic interactions between proteins may be also possible, especially under heating conditions.

The drastic peak viscosity suppression observed in potato starch (which possesses negative charges due to the presence of phosphate monoester group) implied that surface electrostatic interactions of starch granules with carom proteins could be the reason for the observed viscosity supressing effect. However, it remains inconclusive why other protein sources (such as sodium caseinate and whey protein) reported in other studies do not show similar drastic suppressing effects (Considine et al., 2011).

7.3.6.2 Morphology of different starch granules using light microscopy

Figures 45 to 50 show the effect of carom extract on the morphological changes of tapioca, sweet potato, potato, wheat, maize, and pea starch granules during heating.

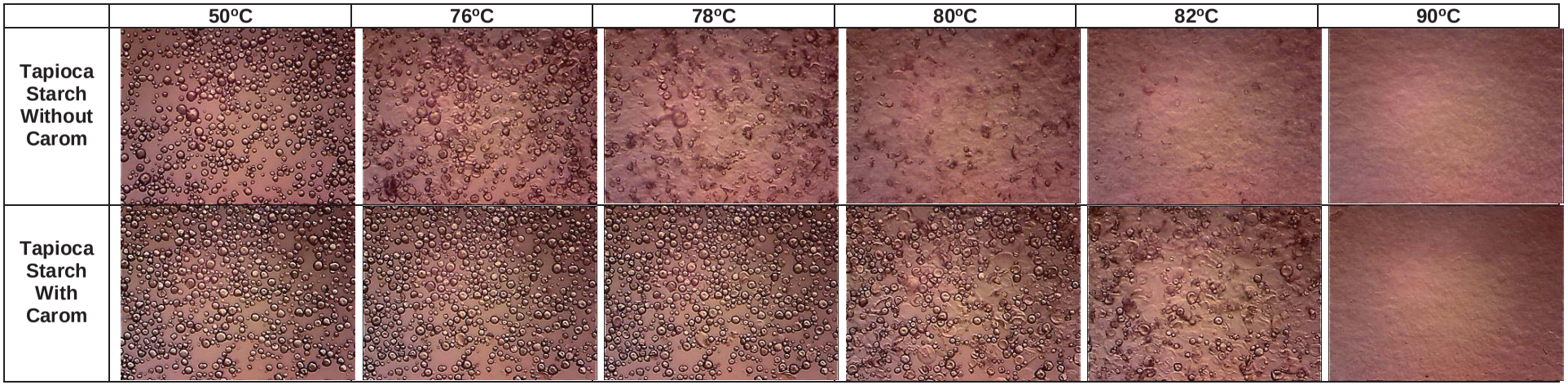


Figure 45: Hot stage micrographs of 1.5% (w/w) tapioca starch with and without 3.5% (w/w) freeze-dried carom extract dispersed in water was heated from 50°C to 90°C at the rate of 19 °C/min.

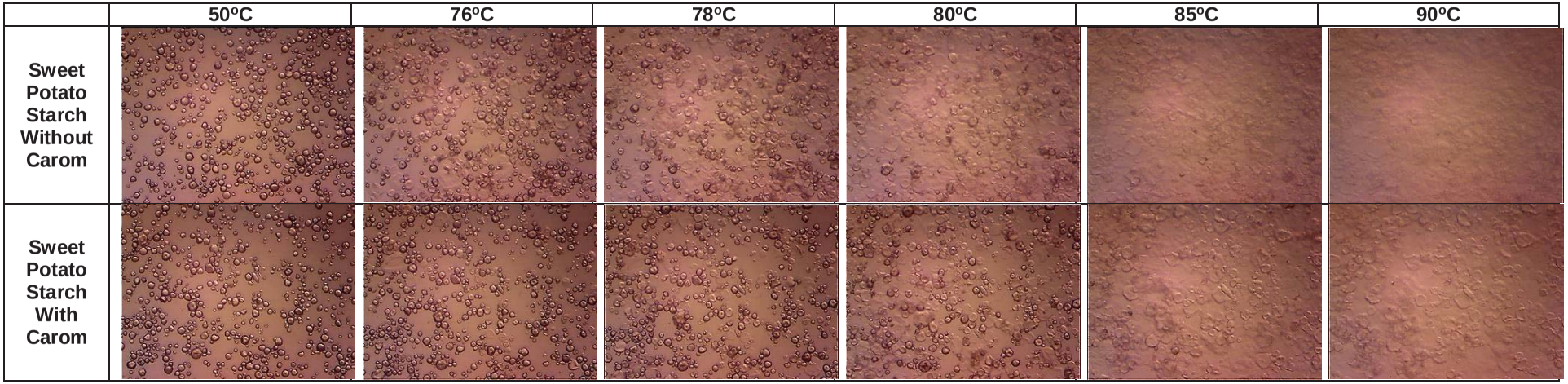


Figure 46: Hot stage micrographs of 1.5% (w/w) sweet potato starch with and without 3.5% (w/w) freeze-dried carom extract dispersed in water was heated from 50°C to 90°C at the rate of 19 °C/min.

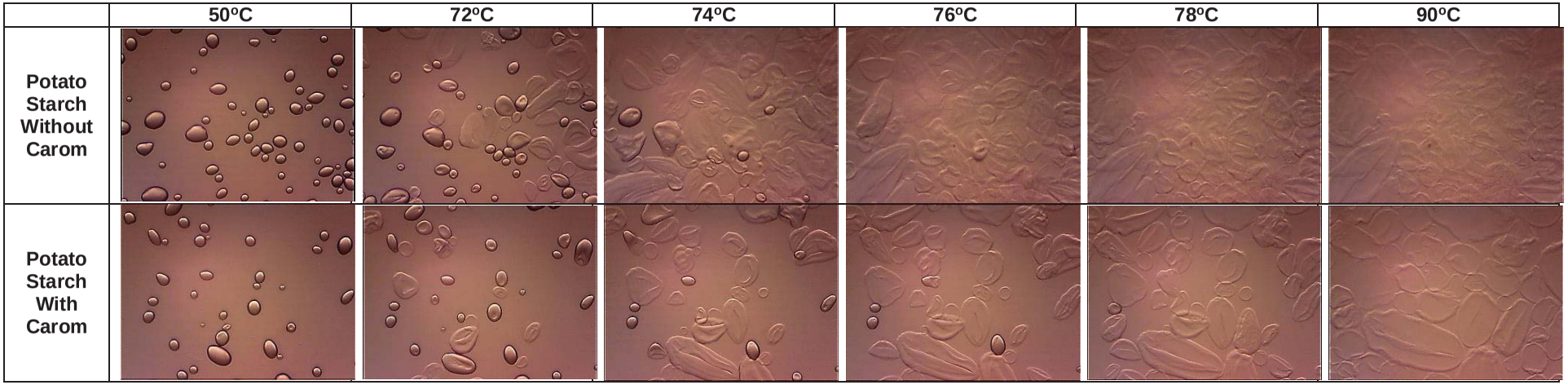


Figure 47: *Hot stage micrographs of 1.5% (w/w) potato starch with and without 3.5% (w/w) freeze-dried carom extract dispersed in water was heated from 50°C to 90°C at the rate of 19°C/min.*

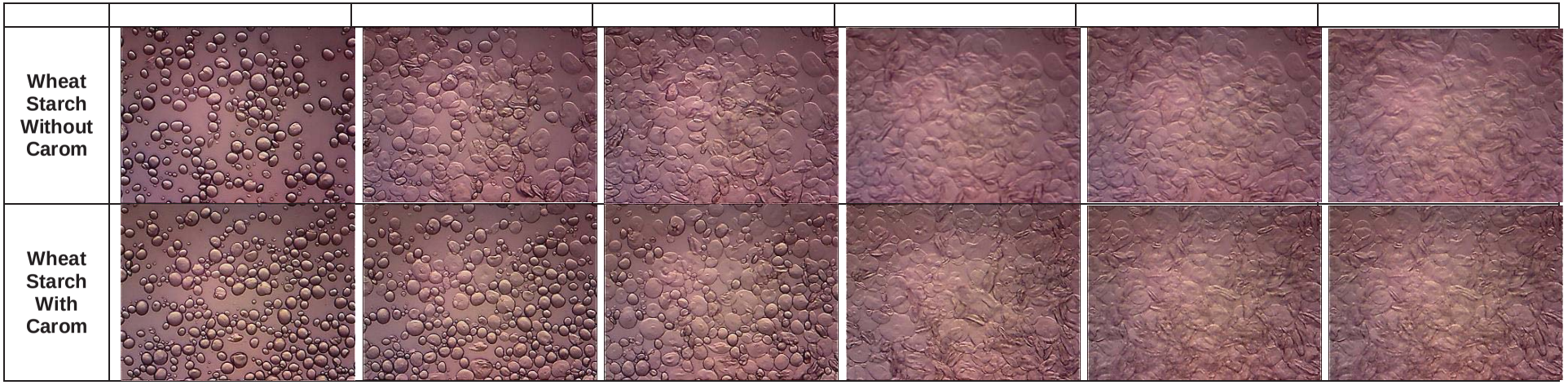


Figure 48: *Hot stage micrographs of 1.5% (w/w) wheat starch with and without 3.5% (w/w) freeze-dried carom extract dispersed in water was heated from 50°C to 90°C at the rate of 19°C/min.*

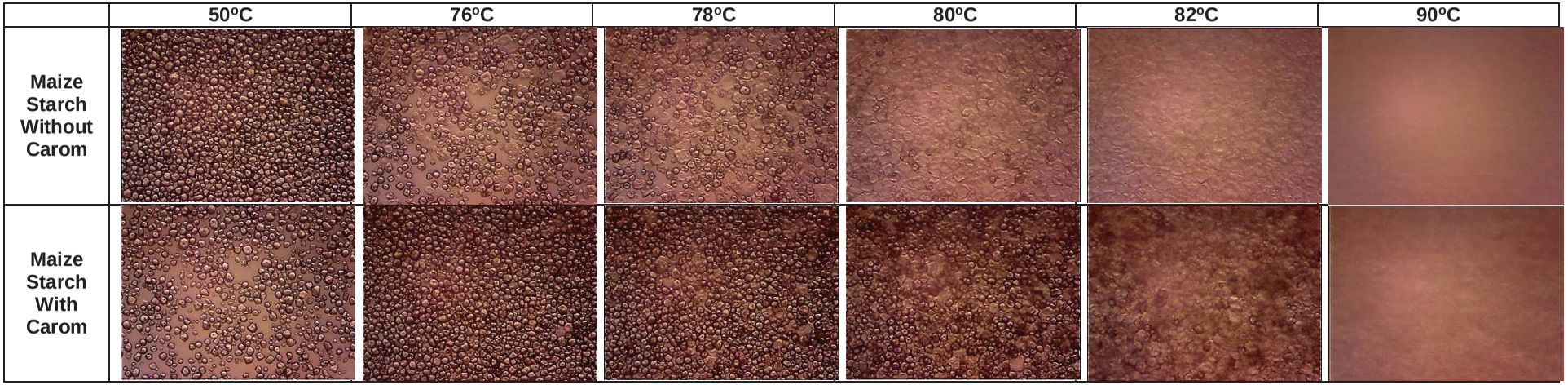


Figure 49: *Hot stage micrographs of 1.5% (w/w) maize starch with and without 3.5% (w/w) freeze-dried carom extract dispersed in water was heated from 50°C to 90°C at the rate of 19 °C/min.*

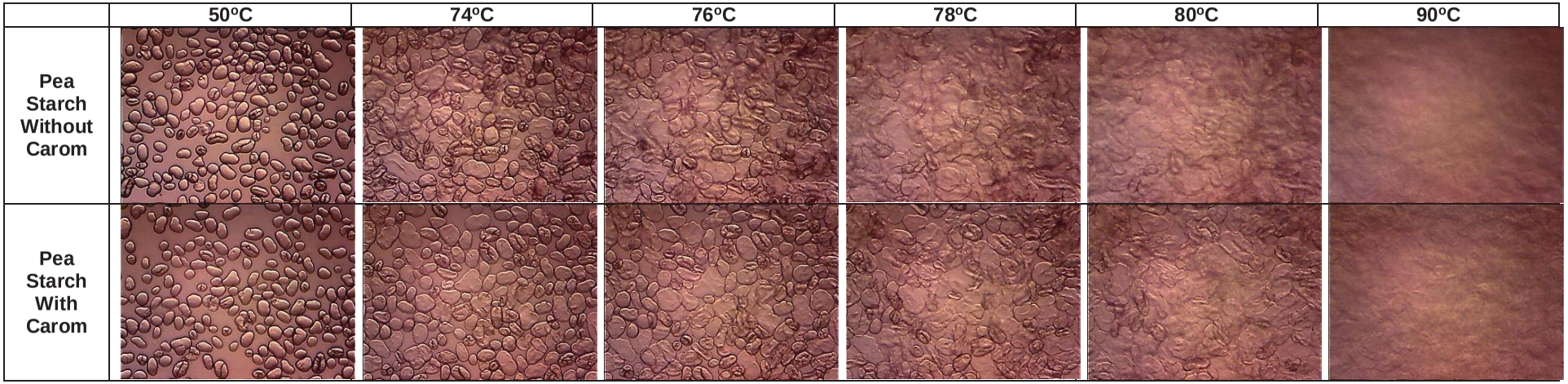


Figure 50: *Hot stage micrographs of 1.5% (w/w) pea starch with and without 3.5% (w/w) freeze-dried carom extract dispersed in water was heated from 50°C to 90°C at the rate of 19 °C/min.*

The presence of dialysed carom extract appeared to restrict the swelling of the respective starch granules. The effect seem more prominent in root and tuber starches due to the larger granular sizes. However, root, tuber, and cereal starches displayed restriction and delay in granular swelling. All the starches ultimately swelled at $\geq$ 90^{o}C. There are currently insufficient data on the effect of protein on starch granular swelling or morphological changes. However, the presence of macromolecules or small molecular compounds (e.g. sugars, salts, and phytochemicals) are known to influence the thermal and rheological properties of starch, and may similarly influence the structural properties observed.

7.3.7 Morphology of rice starch-carom mixtures examined by SEM

The morphology and microstructure of rice starch (with and without carom) at different temperatures were examined using scanning electron microscopy (SEM). The samples for SEM micrographs are based on the pasting process carried out using the rheometer. Figure 51 shows the pasting curve of rice starch with and without carom extract. The graph indicates five temperatures (50^{o}C, 72^{o}C, 85^{o}C, 95^{o}C and 25^{o}C) which are the sampling points at which aliquots were obtained for SEM examination. Figure 52 shows the SEM micrographs (10 and 20µm) of these five respective temperature points and an additional point that involved chilling the final gel at 5^{o}C for 24 hours.

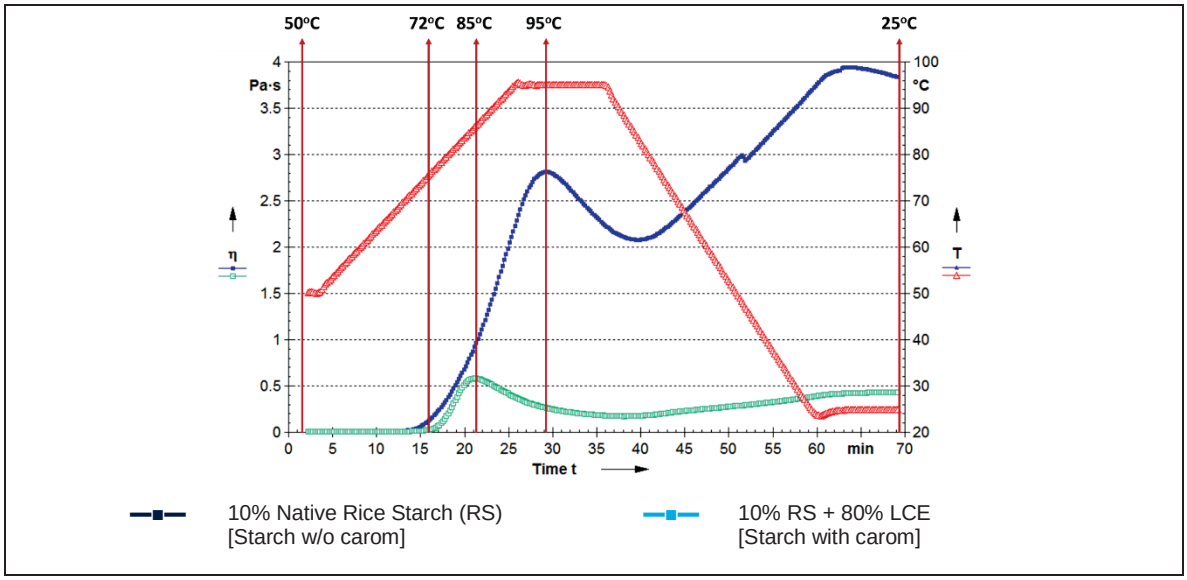


Figure 51: *The pasting curve of 10% (w/w) rice starch (RS) with and without 80% (w/w) liquid carom extract (LCE), with six temperature points (50^{o}C, 72^{o}C, 85^{o}C, 95^{o}C, 25^{o}C, and 5^{o}C) at which aliquots were sampled for SEM analysis.*

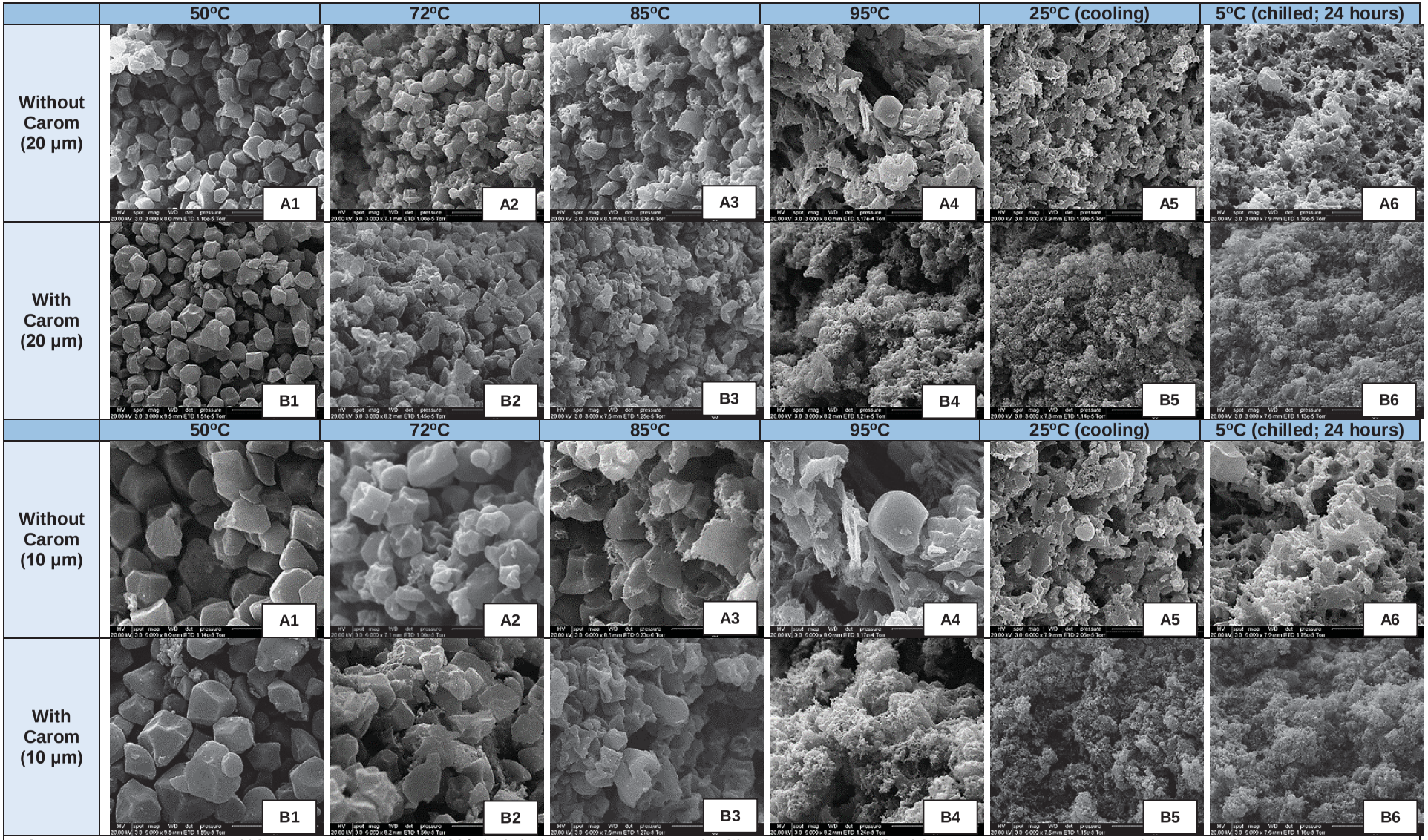


***Figure 52: SEM** images (10 µm and 20 µm) of 10% (w/w) rice starch with and without 80% (w/w) liquid carom extract dispersed in water; heated from 50°C to 95°C at the rate of 2°C/min and held at 95°C for 10 mins and cooled from 95°C to 25°C at the rate of 3°C/min, followed by chilling for 24 hours at 5°C.*

At 50ºC, no difference was observed between samples with and without carom (A1 and B1 in Figure 52) since starch granules remained intact and had not undergone swelling. The effects of the addition of carom extract were not visible under SEM at this stage. This observation is consistent with rheological data, which showed no change on viscosity at 50ºC until the onset temperature.

At 72ºC, which is the onset temperature for rice starch with carom, the integrity of the starch granules appeared to be slightly different with slight swelling and indentations as compared to the starch granules at 50ºC. However, there were no noticeable differences in the morphology between starch granules with and w/o carom.

At 85ºC, the SEM images show that the starch granules with carom (B3 in Figure 52) were slightly smaller and less swollen as compared to starch without carom. However, there was no drastic differences in the morphology between starch granules with and w/o carom.

The pasting curve (Figure 51) shows that the starch w/o carom reached its peak viscosity at 95ºC. However, for starch with carom sample, the peak viscosity had already occurred and was decreasing towards the holding strength point. At this temperature, a vast difference in viscosity was obtained for rice starch with and without carom (Figure 51). The SEM images show that the starch w/o carom (A4 in Figure 52) appeared to have lost most of its granular shapes. Instead, larger and flatter structures were mainly observed, which could be from the partially ruptured starch granules. Interestingly, the starch with carom (B4 in Figure 52) appeared very differently. The granules seemed to be engulfed by clusters of aggregates, which are believed to be the denatured carom protein as observed in Figure 34. The aggregated carom proteins were possibly surrounding the starch granules, thereby restricting granules swelling leading to the observed delay in starch gelatinisation during the pasting process.

After cooling to 25°C, the starch paste developed a distinct gel-like properties as linear amylose molecules formed extensive hydrogen bonds to create a three dimensional network that trapped water and increased the rigidity of the starch mass

(A. Brown, 2008; Yook et al., 1993). The pasting curve (Figure 51) shows that the rice starch without carom had a final viscosity that was higher than the peak viscosity. However, rice starch with carom had a very low final viscosity.

SEM images show that rice starch without carom (A5 of Figure 52) had a continuous interconnected structure, resembling a typical porous network. However, rice starch with carom (B5 of Figure 52) showed structures with clusters of aggregates without a defined network. This difference is further emphasized in A6 and B6, after storing the gels at 5°C, where the structures could be described as coral versus broccoli in the absence or in the presence of carom respectively.

7.4 Proposed Mechanism

The presence of carom protein has led to the suppression of peak and final viscosities of different starches during the pasting process. To date, no proteins found in the literature have a profound viscosity suppressing effect similar to carom protein. Based on the rheological, thermal, and microstructural data, a mechanism for starch-carom protein interactions that leads to the observed viscosity suppressing effect can be proposed. A schematic of this proposed mechanism is shown in Figure 53.

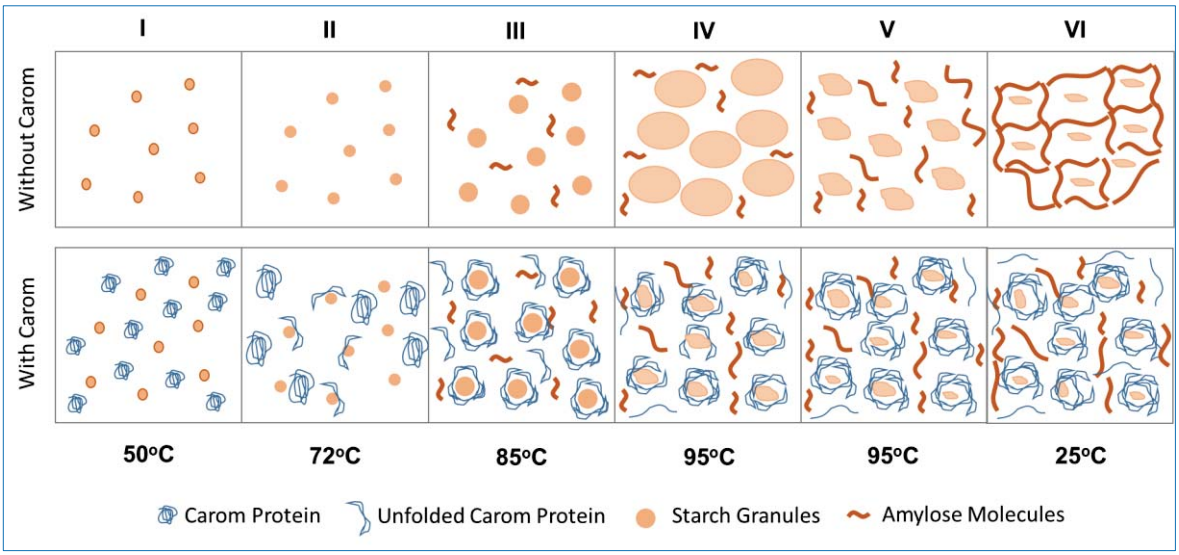


***Figure 53**: A schematic diagram of the proposed mechanism of the starch-carom system.*

From 50 to 72°C, carom protein molecules are well dispersed among starch granules (I in Figure 53). From 72 to 85°C, starch granules started to swell. At the same time, carom protein molecules begin to unfold and could start to interact with the surfaces of starch granules (II in Figure 53). The interactions between carom proteins and starch granules could involve several possible interactions including hydrogen bonds, electrostatic interactions between carom proteins and proteins and phospholipids on the surfaces of starch granules, and hydrophobic interactions between carom proteins and proteins found on starch granules. From 85 to 95°C, carom proteins begin to form aggregates and can deposit on the surfaces of the starch granules (III & IV in Figure 53). Starch swelling is restricted due to the presence of carom protein aggregated layer on starch granules, restricting the diffusion of water into the starch granules and delays the initialisation or onset of gelatinization. Prolonged heating at 95°C can lead to more unfolding and aggregation of the carom protein on the starch surfaces. At the elevated temperature, swelling of starch granules still occurs but within the aggregated protein, which disrupts the continuous starch, granules network (V in Figure 53). When the paste is cooled to 25°C, the amylose that leaches out is hindered by the presence of carom protein to form a gel network (VI in Figure 53).

The amount of literature available to elucidate this interaction between proteins with starch granules is limited. However, studies have shown that the proteins present in sodium caseinate and whey protein isolate are adsorbed onto native and waxy rice starch granules (Considine et al., 2011; Noisuwan et al., 2011). The surface of the starch granule is complex, as it consists of proteins and lipids (Baldwin, 2001). Studies have shown that polar lipids and puroindoline (lipid binding protein) found on the surface of wheat starch were necessary for maximum adhesion of added soy protein fractions to the exterior of starch granules (Ryan & Brewer, 2005a, 2005b). The authors have shown that the removal of the wheat starch granular surface protein resulted in a decrease in the binding of added proteins, suggesting that native granule proteins might mediate the binding of exogenous protein. Therefore, it is plausible that the adsorption of the carom proteins onto starch granules is mediated by these indigenous starch surface granular lipids and proteins.

7.5 Conclusion

It was conclusive that the protein fraction in carom extract was responsible for the viscosity-suppression effect of rice starch during pasting. The carom protein had an M_w of ~2.08 ± 0.10 x 10^5 Da and an isoelectric point of ~3.5. The actual protein fraction responsible for suppressing the viscosity of starch during gelatinisation appeared to be denatured at approximately pH 3. The carom protein was not heat stable above 70^{o}C and was completely denatured at above 83^{o}C. The carom protein had an acidic nature with a minimum solubility at low pH (3 - 4) and maximum solubility at alkaline pH. Observation of SEM micrographs showed that carom protein had a unique raisin-like cluster appearance.

Carom protein was capable of suppressing the viscosity of different starches (potato, tapioca, glutinous rice, waxy maize, waxy rice, rice, sweet potato, maize, wheat, and pea) during the pasting process but to different degrees. The viscosity suppressing effects of carom protein were not dependent on the amylose and amylopectin content.

It was proposed that the carom protein molecules might be unfolding and interacting with the surfaces of starch granules during gelatinization. These interactions may be restricting starch swelling and delaying the initialisation or onset of gelatinization. In addition, the amylose that leaches out may also be hindered by the presence of carom protein that resulted in a weak gel network (low viscosity).

CHAPTER 8: EFFECT OF CAROM ON STARCH DIGESTION

8.1 Introduction

The previous chapter identified that the protein fraction in carom extract was the component inducing viscosity-suppressing effect during starch pasting. This chapter reports on the effect of dialysed carom extract on the digestion rate of starch using an *in-vitro* digestion model. The *in-vitro* model studied the differences between the rate of glucose release of rice starch and rice starch-carom mixtures heated at (i) 72^{o}C for 30 seconds and (ii) 95^{o}C for 10 minutes.

8.2 Materials and Methods

This *in-vitro* digestion model studied the effect of dialysed carom extract on the rate of glucose released during starch breakdown. Dialysed carom extract was prepared based on the procedures outlined in *Section 7.2.2*. Rice starch sample (15.00 g) was prepared using 10.0% (w/w) native rice starch and 90.0% (w/w) Milli-Q water. Rice starch with carom sample (15.00 g) was prepared using 10% (w/w) native rice starch, 10% (w/w) Milli-Q water, and 80% (w/w) dialysed liquid carom extract. Both samples were heated in the rheometer using two different temperature settings. In the first setting, the samples were heated from 50.0^{o}C to 95.0^{o}C ± 0.1^{o}C at 2.0^{o}C per min and held at 95.0^{o}C ± 0.1^{o}C for 10 mins at continuous stirring rate of 160 rpm. The heated samples were then cooled from 95.0^{o}C to 25.0^{o}C ± 0.1^{o}C at 3.0^{o}C per min. The amount of water evaporated during heating was compensated to ensure that the final weight of the sample was 15.00 g. In the second setting, the samples were heated from 50.0^{o}C to 72.0^{o}C ± 0.1^{o}C at 2.0^{o}C per min and held at 72.0^{o}C ± 0.1^{o}C for 30 seconds at continuous stirring rate of 160 rpm. The heated samples were then cooled from 72.0^{o}C to 25.0^{o}C ± 0.1^{o}C at 3.0^{o}C per min. The amount of water evaporated was also compensated.

All four samples were then subjected to an *in-vitro* digestion process. Figure 54 shows the protocol of the *in-vitro* digestion based on the procedures outlined by Hardacre et al. (2015), Goh et al. (2015), and Mishra & Monro (2009).

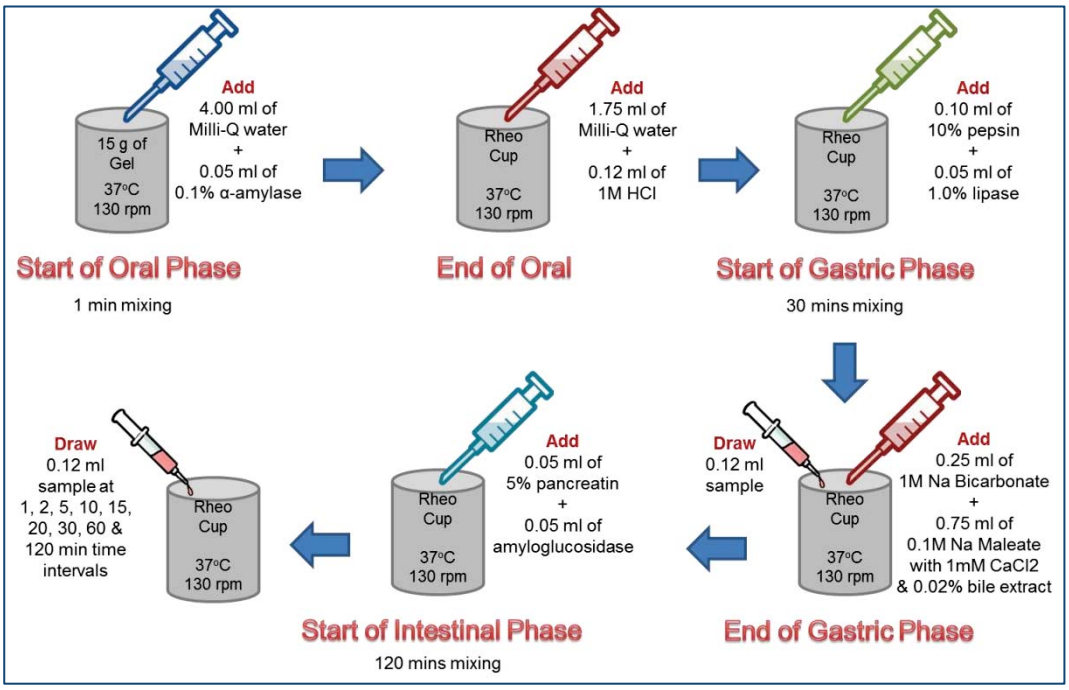


Figure 54: *In-vitro gastrointestinal digestion model*

The *in-vitro* digestion was carried out at 37.0°C ± 0.1°C and at 130 rpm in a starch cell using the MCR rheometer 302 (Anton-Paar, Graz, Austria). Milli-Q water (4 mL) and starch sample (15.00 g) were dispensed into the starch cell and allowed to calibrate for 2 minutes at 37.0°C ± 0.1°C.

The oral phase (R. Goh et al., 2015) was initiated by the addition of 0.05 mL of 0.1% (w/w) α-Amylase (A6255, ≥1000 units/mg protein, Sigma-Aldrich Corporation, USA). The sample was mixed at 37.0°C ± 0.1°C and at 130 rpm for 1 minute.

After 1 minute, the gastric phase (Hardacre, Yap, Lentle, & Monro, 2015) was initiated by the addition of 1.75 mL of Milli-Q water and 0.12 mL of 1M HCl to ensure that the pH was approximately 2.5 $\pm$ 0.1. This was followed by the addition of 0.10 mL of 10.0% (w/w) pepsin (P7000, ≥250 units/mg, Sigma-Aldrich Corporation, USA)(note that pepsin was prepared by dissolving the powder in 0.05 M HCl) and 0.05 mL of 1.0% (w/w) lipase (L3126, 200 units/g, Sigma-Aldrich Corporation, USA). The sample was mixed at 37.0°C ± 0.1°C and at 130 rpm for 30 minutes.

After 30 minutes, the intestinal phase (Hardacre et al., 2015) was initiated with the addition of 0.25 mL of 1M sodium bicarbonate solution and 0.75 mL of 0.1M sodium malate [containing 0.02% (w/w) sodium azide, 1mM CaCl2 and 0.02% (w/w) bile

extract]. The sample was mixed at 37.0^{o}C ± 0.1^{o}C and at 130 rpm for 30 seconds. Then 0.12 mL of aliquot was drawn and added to 0.42 mL of chilled absolute ethanol and mixed thoroughly to halt digestion.

The amylolysis stage (Hardacre et al., 2015) was initiated with the addition of 0.05 mL of 5.0% (w/w) pancreatin (P7545, 8xUSP, Sigma-Aldrich Corporation, USA)(note that pancreatin was prepared by dissolving the powder in 0.1 M sodium maleate) and 0.05 mL of Amyloglucosidase solution (A7095, ≥300 units/mg, Sigma-Aldrich Corporation, USA). The sample was continuously mixed at 37.0^{o}C ± 0.1^{o}C and at 130 rpm. Then 0.12 mL of aliquots were drawn at 1, 2, 5, 10, 15, 20, 30, 60 and 120th min and each was added to 0.42 mL of chilled absolute ethanol and mixed thoroughly to halt digestion. The aliquots were kept at – 20^{o}C for glucose determination.

The amount of sugar released during *in-vitro* digestion was measured as monosaccharides using a modified dinitrosalicylic acid (DNS) colorimetric method based on the procedure outlined by Mishra and Monro (2009). Briefly, aliquot samples were centrifuged at 10, 000 g for 15 minutes at 25°C. Then 0.05 mL of the supernatant was placed into 10 mL test tubes (Kimax, Gerresheimer Glass Inc, Germany) and 0.25 mL of enzyme solution [containing 1% invertase (Fisher Scientific, USA) and 1% amyloglucosidase (E-AMGDF, Megazyme, Ireland)] was added. The tubes were covered and incubated at 37°C ± 1^{o}C using a water bath (WNB 14, Memmert GmbH, Germany) for 10 minutes. The tubes were then transferred into a dark room. This was followed by the addition of 0.75 mL DNS mixture (note that DSC mixture was prepared by dissolving 10.0 g of 3,5-dinitrosalicylic acid in 1 L of solution containing 300.0 g potassium sodium tartrate and 16.0 g sodium hydroxide). The text tubes were covered and incubated to 95°C ± 2^{o}C using the water bath for 15 minutes. The samples were then cooled for 15 minutes at 25°C, followed by the addition 4 mL Milli-Q water. Samples were thoroughly mixed prior to absorbance readings at 530 nm using the visible range spectrophotometer (Thermo Fisher Scientific Inc, USA). The *in-vitro* digestion were conducted in duplicates and the mean value of the two measurements were reported.

The amount of glucose released was calculated based on the calculation outlined by Hardacre et al. (2015). A glucose standard curve using different concentration of

glucose was prepared and the standard curve was plotted. A linear line with a formula of y = 0.0912x (R^2 = 0.992) was obtained as the standard curve for glucose, where y was the optical density (spectrometer) and x was the glucose concentration (mg/ml). The amount of glucose released during the *in vitro* digestion was determined using the equation (1):

$$Reducing\ sugar = \frac{[(OD \times k) \times V]}{(W)\ starch} \quad (1)$$

where OD is the optical density (spectrometer); *k* is the conversion factor (OD to glucose mg/mL) from the standard curve for glucose; V is the total volume of sample (mL); and W is the weight of starch initially present (g).

8.3 Results and discussion

8.3.1 In-vitro starch digestion

Rice starch with and without dialysed carom extract were cooked at two temperatures, namely 72°C (30 seconds) and 95°C (10 minutes) with the aim of studying the effect of carom in influencing glucose assimilation at different heating conditions. The two cooking settings were to mimic pasteurization and high heat processing conditions respectively. The rates at which the starch samples and starch-carom samples were digested were determined indirectly from the rates at which glucose was liberated during amylolysis (Hardacre, Lentle, Yap, & Monro, 2016; Hardacre et al., 2015). Figure 55 shows the amount of glucose released during the *in-vitro* digestion.

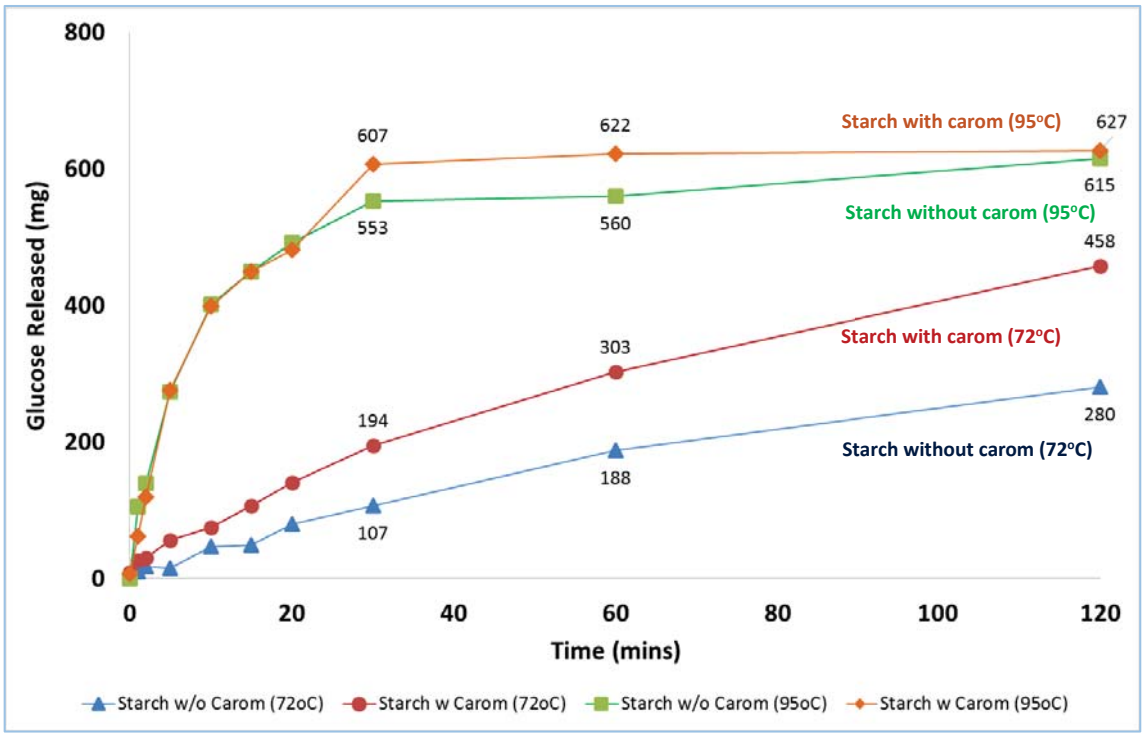


Figure 55: *The rate of glucose released (average of duplicates) during in-vitro starch digestion of rice starch samples and rice starch-carom samples prepared at two different temperature settings.*

Figure 55 shows that a sharp increment was observed for sample prepared at 95°C and a gradual increment for sample prepared at 72°C. The presence of dialysed carom extract in the rice starch sample increased the amount of glucose released. The starch-carom sample (heated at 95°C) started to plateau at the 30th minute and reached the maximum glucose amount (627 mg) at the 60th minute. However, the starch sample without carom (heated at 95°C) gradually reached the maximum

glucose amount (615 mg) at the 120th minute. The starch-carom sample (cooked at 72^{o}C) reached the maximum glucose amount (458 mg) at the 120th minute, while starch sample without carom only reached a maximum of 280 mg at the 120th minute.

A number of factors can influence the amount of glucose released during carbohydrate digestion, including: structure of the starch component, viscosity of the system, presence of resistant starch, the degree of starch damage through processing, and particle size of the food (Aarathi, Urooj, & Puttaraj, 2003; M. A. Brown, Storlien, Brown, & Higgins, 2003; Burke, Collier, & Hargreaves, 1993; Burton, Monro, Alvarez, & Gallagher, 2011; Giacco et al., 2001; Hardacre et al., 2016; Hardacre et al., 2015; Hoebler, Karinthi, Chiron, Champ, & Barry, 1999; Parada & Aguilera, 2009; Tosh & Chu, 2015; Widanagamage et al., 2013).

At 72°C, the degree of starch swelling and amount of amylose molecules leaching out could be possibly lower than the starch heated at 95°C for 10 minutes. Therefore, a high rate of glucose release was observed for samples heated at 95^{o}C as compared 72°C due to a better accessibility of the amylase to hydrolyse the fully gelatinized starch molecules.

Contrary to expectation, the starch gel without carom had a lower rate of glucose release as compared to starch-carom sample. This effect was reproducible and was observed for samples heated at both 72°C and 95^{o}C. This unique effect maybe contributed due to (i) larger surface area exposure for amylase to hydrolyse, (ii) lower viscosity of the starch-carom sample, or (iii) enzyme stimulatory effect of carom extract.

One of the possible explanation could be due to the effect of carom protein depositing on the surfaces of starch granules (refer to *Section 7.3.7*). As a result, starch molecules in the presence of carom did not have the opportunity to retrograde and form 3-D network to the same extent as gelatinised starch alone. The poor network formation may have led to a low final viscosity of starch-carom sample (Figure 51). Therefore, the characteristically smaller starch granules and free amylose molecules that did not undergo retrogradation would have a larger surface exposure for pancreatic amylase to hydrolyse the starch during digestion.

It was evident that starch-carom samples usually had a drastically low peak and final viscosities as compared to starch alone (Figure 51). The lower viscosity would mean more efficient enzyme-substrate interaction. Therefore, the larger surface area of starch granules, a lower degree of retrogradation, and a lower viscosity of starch-carom sample could have facilitated an increased amount of glucose release.

Another possible explanation could be due to the enzyme stimulatory effect of carom (acting like a catalyst) that could have increased the activity of pancreatin (pancreatic amylase, lipase, and protease) and/or amyloglucosidase. In fact, several *in vitro* and *in-vivo* animal studies have shown that carom extract increased the activities of pancreatic and intestinal enzymes (e.g. lipase and amylase) significantly (Platel et al., 2002; Platel & Srinivasan, 1996, 2000a, 2000b, 2001, 2004; R. R. Rao, Platel, & Srinivasan, 2003; Srinivasan, 2005b). In one study, it was reported that the pancreatic lipase activity increased by 26%, pancreatic amylase by 9%, chymotrypsin by 5%, and trypsin by 48% with the continued intake of carom. In addition, the intestinal lipase activity increased by 113% and intestinal amylase by 74% with one-time exposure of carom (Platel & Srinivasan, 2001). Similarly, in an *in-vitro* study, carom extract in saline solution showed enhanced pancreatic enzyme activities of lipase and amylase when present at the site of enzyme action (R. R. Rao et al., 2003). However, the exact mechanism for the enhancement of pancreatic and intestinal enzyme activities were not explained by the authors. To date, this was the first *in vitro* digestion study to observe that carom extract increased the rate of starch hydrolysis. This study would add on new information to existing preliminary scientific literature and ethno-medicinal claims on carom's digestive stimulatory action. However, further *in vitro* and *in vivo* studies are required to understand this mechanism further.

Studies have shown that spices and herbs may contain naturally occurring amylases that may hydrolyse starch (Nomura et al., 1999). However, the presence of natural amylases in carom extract possibly inducing the viscosity-suppressing effect via enzymatic hydrolysis seemed unfounded. This is because the presence of natural amylases would have hydrolysed the starch samples and would have released certain amount of glucose at the 0^{th} min, which was not observed in Figure 55.

8.3.2 Potential nutritional applications

Based on the preliminary *in-vitro* data from this study, carom extract could potentially be incorporated into mixed meals or products that are high in carbohydrate to increase the rate of starch digestion and glucose assimilation. These modified meals or products may be useful for individuals such as athletes and patients with pre-existing digestive disorders. It is widely documented that athletes should consume high glycemic index foods during and after exercise (Burke et al., 1993; Walton & Rhodes, 1997; Wright, 2005). These foods will ensure rapid digestion and absorption, which will lead to elevated blood glucose levels (Walton & Rhodes, 1997). High blood glucose levels are important for muscle glycogen resynthesis (Betts & Williams, 2010; Burke et al., 1993; Carlsohn & Mayer, 2010; Kaye & Brand-Miller, 2011). Patients with compromised digestive system or gastro-intestinal disorder (e.g. indigestion) could also benefit from the rapid breakdown of carbohydrates during ingestion. However, nutritional intervention studies using human subjects are necessary to verify such postulation.

8.4 Conclusion

The presence of dialysed carom extract in the rice starch samples increased the amount of glucose released. The unusual increment in glucose release was attributed to the low viscosity of the sample or to an enzyme stimulatory effect of carom. The low viscosity of the starch-carom gels may significantly increase the enzyme-substrate interaction for hydrolysis. In addition, carom was also reported to have enzyme stimulatory effect that could increase the activity of pancreatic amylase to breakdown starch.

CHAPTER 9: OVERALL CONCLUSION & RECOMMENDATIONS

9.1 Conclusion

Seven spice and herbal water extracts (carom, cumin, fennel, mulberry leaf, perilla leaf, neem and coriander seed) showed peak and final viscosity-suppressing effect, while five (mesona, rosemary, green tea, thyme and clove) showed peak viscosity-enhancing effect on 10% (w/w) native rice starch during starch pasting. Water-soluble fraction of carom had the highest degree of viscosity-suppressing effect as compared to other spices and herbs. To date, this was the first study to report on such findings.

Out of all the components (such as proteins, polysaccharides, lipids, acids, bases, ions, sugars, phytochemicals, or naturally occurring amylases) that could potentially interact with starch, proteins were identified as the likely fraction responsible for the viscosity suppression effects observed specifically in starch-carom system.

With increasing concentration of carom extract, the peak and final viscosities of rice starch decreased; onset, peak, and end temperatures of rice starch increased; and granular swelling of potato starch was restricted and delayed.

It was conclusive that the viscosity-suppressing effect was not caused by pH or small molecular carom compounds such as mineral salts (e.g. calcium chloride, potassium chloride, sodium chloride, magnesium chloride, sodium phosphate, and ferrous gluconate) and phytochemicals (e.g. thymol, ρ-cymene, S-limonene, γ-terpinene, saponins, and tannic acid). Instead, the protein polymer in carom extract was deduced to responsible for the drastic viscosity suppression of starch. The protease treatment of carom extract for 1 hour and 24 hours reduced the M_w of a polymer fraction (observed as a reduction in the light scattering peak) that correlated to the loss of viscosity-suppressing effect in the pasting curves.The carom protein had an M_w of ~2.08 $\pm$ 0.10 x 10^5 Da and isoelectric point of ~3.5. The protein fraction responsible for suppressing the viscosity of starch during pasting diminished at `pH 3 and at temperature above 80^{o}C. Observation of SEM micrographs showed that carom protein had a unique raisin-like cluster appearance.

The protein in carom extract was not influenced by the type and ratios of amylose to amylopectin content as the peak viscosities of different starches (potato, tapioca, glutinous rice, waxy maize, waxy rice, rice, sweet potato, maize, wheat, and pea) showed similar reduction during starch pasting.

It was proposed that the carom protein molecules might unfold and interact with the surfaces of starch granules during gelatinization. The interactions between carom proteins and starch granules could involve several possible interactions including (i) hydrogen bonds, (ii) electrostatic interactions between carom proteins and proteins and phospholipids on the surfaces of starch granules, and/or (iii) hydrophobic interactions between carom proteins and proteins found on starch granules. These interactions may restrict starch swelling (low peak viscosity) as the diffusion of water into the starch granules may be hindered. In addition, it is also proposed that the amylose that leaches out is hindered by the presence of carom protein resulting in a weak gel network (low final viscosity).

The presence of dialysed carom extract in the rice starch gels unexpectedly increased the amount of glucose released. The unusual increment in glucose release was attributed to the low viscosity of the gel and possibly to an enzymatic stimulatory effect of component(s) found in the carom extract.

9.2 Recommendation

The following are recommendations for future studies:

- Components in different spices and herbs

The components in the spice and herbal extracts that were responsible for the viscosity suppression or enhancement are to be identified and characterised. The basic chemical composition of all the extracts and their effects on the rheological, thermal, and structural properties and digestibility of starches to be explored.

- Effect of naturally-occurring amylases

Studies have shown that spices and herbs may contain naturally occurring amylases that may hydrolyse starch (Nomura et al., 1999). The presence of natural amylases in carom extract possibly inducing the viscosity-suppressing effect via enzymatic hydrolysis requires further investigation.

- Effects of other spices and herbs

A wider variety of other spices and herbs from different regions can be screened for their possible effect on the rheological properties and *in vitro* digestibility of starch. These may include unique medicinal herbs used by certain communities such as the Māori, which includes Mānuka (*Leptospermum scoparium*), Kōwhai (*Sophora microphylla*) and Kawakawa (*Macropiper excelsum*). It may also include other uncommon culinary spices and herbs such as Hyssop (*Hyssopus officinalis*), Garden cress (*Lepidium sativum*), and Lavender (*Lavandula angustifolia*).

- Carom protein characterization

The protein found in the carom extract should be isolated and purified, and then characterized to understand its properties. The exact mechanism of the starch-carom protein interaction requires further studies to understand the cause for the drastic viscosity reduction.

- Characterisation techniques

Techniques such as nuclear magnetic resonance (NMR) can expound the interaction (e.g. hydrogen bonding interaction during pasting/ gelatinization) between starch and components of spices and herbs.

- Starch digestion

The effect of the remaining thirty-five different spices and herbs on starch digestion rate should be analysed. The *in vitro* methodology established by different authors can be reviewed and compared to determine whether similar glucose assimilation data are obtained using different methods.

- Carom influence of glucose assimilation

Further studies are required to determine the cause for the rapid glucose release in starch samples with carom extract. The mechanism of protein and other components that influence the digestibility of starch needs further investigation. The digestive stimulating effect of carom extract requires further validation with *in-vivo* studies.

- Nutrition application

The application of carom and other spices and herbs in starch-based foods to increase or decrease the glycemic response in human subjects may be studied. The potential use of carom extract in the development of food products for sport nutrition for rapid glucose release may also be explored.

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APPENDICES

			
Carom	Coriander Seed	Cumin	Fennel
			
Coriander Leaf	Parsley	Dill	Codonopsis Root
			
Chinese Yam	Red Bush Tea	Reishi Mushroom	Mesona
			
Rosemary	Thyme	Holy Basil	Perilla Leaf
			
Mint	Oregano	Sage	Cinnamon

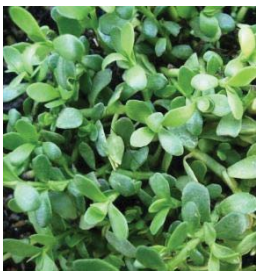
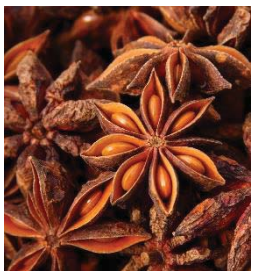
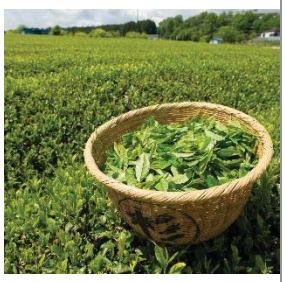
			
Neem	Mulberry Leaf	Clove	Nutmeg
			
Indian Gooseberry	Black Pepper	Brahmi	Loguat Leaf
			
Coffee	Star Anise	Black Tea	Green Tea
			
Oolong Tea	Cardamom	Ginger	Turmeric

Figure A1: *Pictures of the 36 different spices and herbs used in the study. Source: Wikimedia Commons*

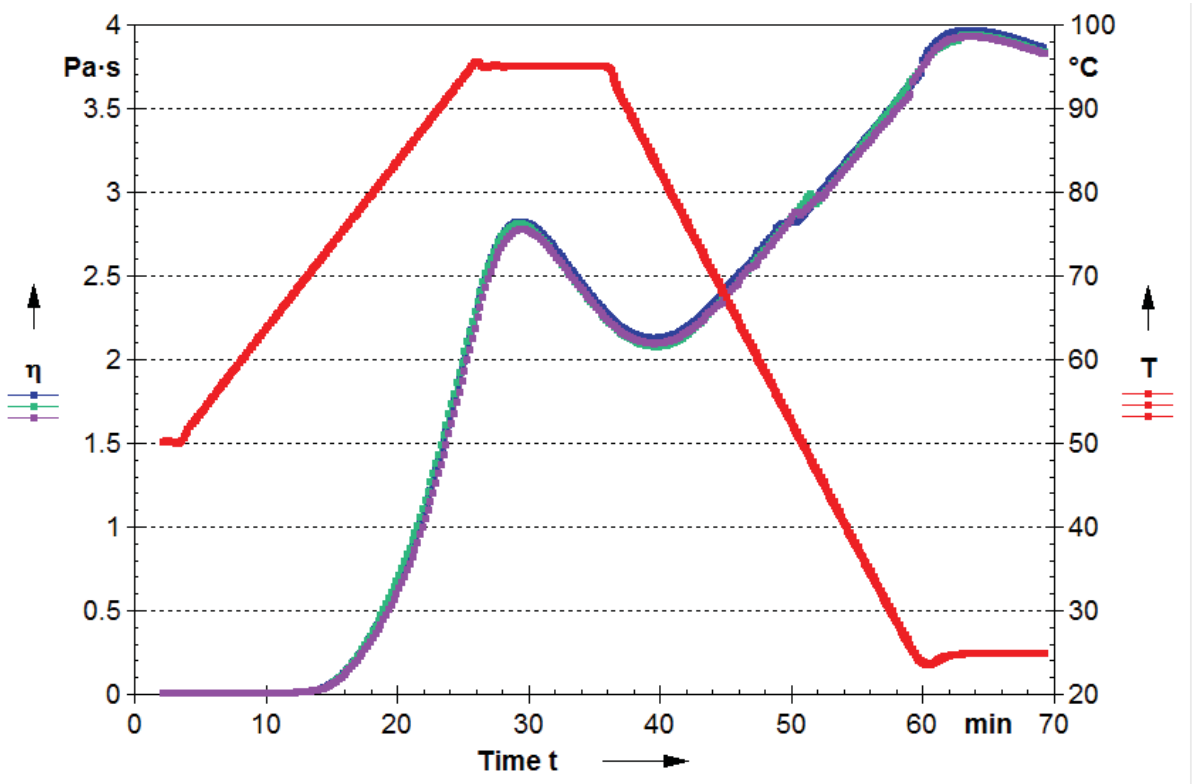


Figure A2: Triplicate measurements of 10% (w/w) native rice starch suspension, which superimposed closely.

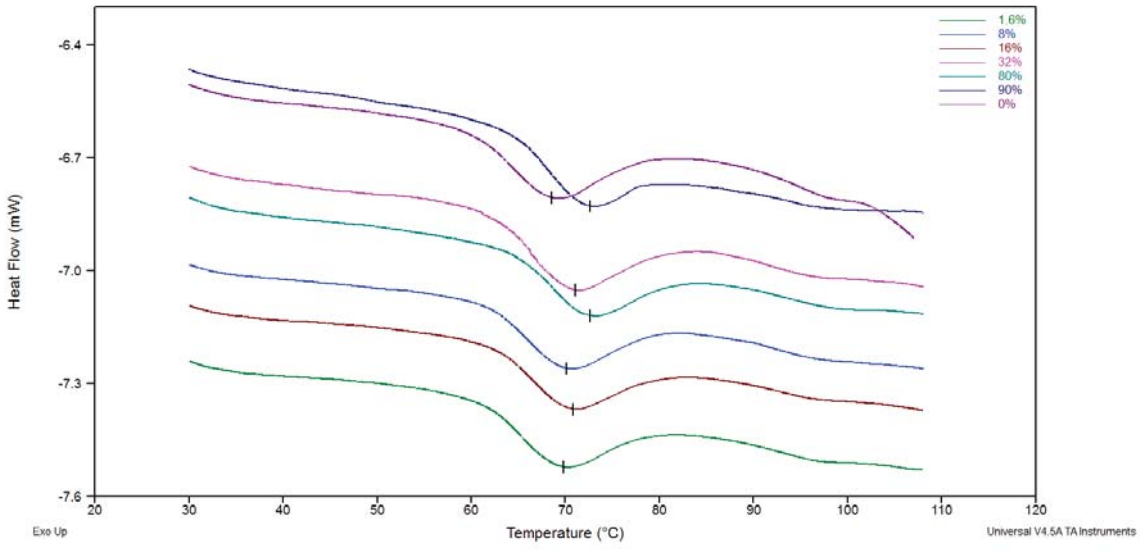


Figure A3: *Differential Scanning Calorimetry endothermic curves of 10% (w/w) native rice starch with varying liquid carom extract concentration (0%, 1.6%, 8%, 16%, 32%, 80% and 90%) (w/w).*