PREPARING OF RESISTANT STARCH WITH HEAT MOITSTURE TREATMENT, ACID MODIFICATION, ENZYMATIC MODIFICATION, AND EPOXIDATION METHODS

By

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TITLE: PREPARING OF RESISTANT STARCH WITH HEAT MOITSTURE TREATMENT, ACID MODIFICATION, ENZYMATIC MODIFICATION, AND EPOXIDATION METHODS

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ABSTRACT

The main objectives of this study were to develop an effective process to produce modified pea starch with enhanced enzyme resistance property (RS) for food applications. The work compares a non-chemical method (heat moisture treatment) versus a chemical method (crosslinking). One type of commercial pea starch (Nutri-Pea) was used exclusively as the raw material in this study. A number of methods were used to characterize the properties of the modified pea starches: water solubility index (WSI), titration (conversion, iodine value), intrinsic viscosity, infrared spectroscopy (FT-IR), Englyst digestion method, total starch content, and rapid visco analysis (RVA).

The effects of heat-moisture treatment on native pea starch and enzyme treated pea starch were examined. The results showed that the produced samples with both native starch and enzyme treated starch exhibited a sharp increase in intrinsic viscosity. Overall, this method was deemed undesirable and not extensively examined past preliminary evaluations.

The main focus of the study was on a citric acid crosslinking reaction, chosen for its food compliancy. A temperature of 120 ^{o}C was considered ideal for the reaction. FT-IR confirmed the presence of the citric acid incorporation in the starch samples. To improve the extent of reaction, Butanetetracarboxylic acid (BTCA) was considered as a replacement for citric acid and its treated samples showed higher conversion and lower water solubility index than that of the citric acid treated samples. Sodium propionate

(NaP) was also considered in the reaction, this time as a food-grade catalyst and found to have minor benefit in cross-linking. BTCA/NaP treated sample reached the highest conversion of the study (96.8±2.3 %) and the lowest WSI (13.1±2.0 %), which increased the RS fraction of the starch from 18% to 32%. The RVA pasting profiles examined were too low to compare due to the high degree of cross-linking.

Further improvements to RS were sought by debranching the starch before acid crosslinking. A BTCA/NaP treated sample with enzyme treatment showed a low WSI (31.7±2.3 %) yet substantially higher RS fraction (80.81±0.18 %). Similar to the non-debranched acid modified samples, there were no significant RVA pasting results because of the high cross-linking.

Finally, crosslinking with an epoxidized oil was tested to continue looking at food-grade solution yet possible increase the rate of the crosslinking reaction. The results of WSI indicated that this method had little influence on cross-linking, possibly due to the low epoxidation efficiency of vegetable oils, as determined by iodine value.

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ABBREVIATIONS

AACC	American Association of Cereal Chemist
AM	Amylose
ANN	Annealing
AOAC	Association of Official Analytical Chemists
AP	Amylopectin
BTCA	1,2,3,4-Butanetetracarboxylic Acid
CA	Citric Acid
CLA	Cross-linking Agent
DP	Degree of Polymerization
DSC	Differential Scanning Calorimetry
ESO	Epoxidized Soybean Oil
FT-IR	Fourier Transform Infrared Spectroscopy
HMT	Heat-moisture Treatment
NaP	Sodium Propionate
NMR	Nuclear Magnetic Resonance
RDS	Rapid Digestible Starch
RS	Resistant Starch
RTD	Residence Time Distribution
RVA	Rapid Visco Analysis
SCFA	Short Chain Fatty Acid
SDS	Slowly Digestible Starch

SSE	Single Screw Extruder
TDF	Total Dietary Fiber
TPS	Thermoplastic Starch
TSE	Twin Screw Extruder

CHAPTER 1. INTRODUCTION

1.1 Background

Canada is the second largest producer of pulse crops overall and the greatest producer of peas. With growing consumer demand for pulse products, the value of Canada's pulses industry is worth more than one billion dollars per year (Hoover et al. 2010). Therefore, the Canadian food industry is increasingly looking for viable methods to improve the functional and nutritional properties of pulses in order to increase their value as well as capitalized upon their diverse health benefits. Simultaneously with growing pulse interest, over the recent decade there has been considerable interest for resistant starch (RS) due to its unique physiological effects and health benefits. Resistant starch plays an important role in the digestive system where its fermented in the large intestine produces short chain fatty acids (SCFA) such as acetates, butyrates, and propionates. The advantages of these short chain fatty acids include the following major benefits; (Brouns et al. 2002; Pinky et al. 2015; Rekha et al. 2006)

- Stimulate blood flow to the colon
- Increase nutrient circulation
- Help absorb minerals
- Help prevent from absorbing toxic/carcinogenic compounds
- Help to lower blood cholesterol and fats

- Help us feel full (Better satiety)
- Improve insulin sensitivity

To look at methods of creating resistant pulse starch ingredients for food, this study is a part of Pulse Science Cluster 2 which is a large national research program in Canada and it was funded by Agriculture and Agri-Food Canada.

In response to the demands for resistant starch, there have been many studies on making this functional food; however, most have encountered limits on significantly increasing the nutritional resistant content. Common methods to increasing digestion resistant starch include heat-moisture treatment (Uttapap et al., 2012; Chen et al., 2016) and crosslinking (Moon et al., 2007; Malgorzata et al., 2016). Only a small number of studies with pea starch have been reported for this functional food compared to other starches such as rice, potato, and so on. The effect of debranching on different starches (Perry et al., 2009; Pongjanta et al., 2009) offers an alternative approach to resistant starch preparation, though only demonstrated with maize starch so far. Until now, no research has been reported on dual modification for heat-moisture treatment or acid modification using a debranched pea starches as a mean to generate higher resistant fractions than previously reported in the literature.

This thesis will focus on the heat moisture treatment method, and exploring different solution and bulk chemical modification methods of a

pea starch for healthy food applications. The modified starch samples were analyzed by method such as *in-vitro* digestibility (Englyst method), water solubility index (WSI), rapid visco analysis (RVA), and colorimetric titration. The intent was to create a gelatinized materials demonstrating high digestion resistance.

1.2 Research Objectives

The main objective of this study was to develop an effective reaction process done using both solution and bulk modification routes to produce modified pea starches with enhanced RS content as a functional food component. Only one commercial pea starch grade (Accu-Gel^{TM} which was donated by Nutri-Pea Limited) was used in this study. The research included the following major tasks:

- To develop an effective reaction process for native pea starch based on acid or glycidyl crosslinking/branching and compare to samples changed by heat moisture treatment.
- Examine the preparation of an acid modified pea starch using a twin-screw extruder.
- Investigate the effects of debranching enzyme on the functional (RDS, SDS, and RS content, total starch content) and other properties (water solubility index, intrinsic viscosity, conversion, and so on) of modified pea starch samples.

1.3 Thesis Outline

There are five chapters in this thesis. A brief introduction about research background and project objectives are been in Chapter 1. Chapter 2 is a literature review related to the recent advances in studies on structure, properties, characterization, preparation and applications of heat moisture treated starches, acid modified starches, extruded starches, and enzyme modified starches. Chapter 3 describes all materials which were used in this study, preparations, and characterization methods of various modified pea starches. Chapter 4 discusses in detail for all experiments results of pea starch samples prepared by various methods. The conclusions of this study were summarized in Chapter 5. In addition, some recommendations for the future research work are also included in the final chapter.

CHAPTER 2. LITERATURE REVIEW

2.1 Morphology and Structure of Pulse Starches

2.1.1 Chemical Structure of Pulse Starches

Starch is an important food energy source for people and a representative bio-degradable biopolymer in plant life. Starch is composed of two major components, amylose (AM) and amylopectin (AP). Amylose is defined as a linear polymer comprised of glucose units connected by α 1-4 glycosidic bonds with a molecular weight of 10^5-10^6 and has a degree of polymerization (DP) up to 6000, while amylopectin is a highly branched polymer in which many of the glucose units are connected by α 1-4 glycosidic bonds, but, some portions are connected by α 1-6 glycosidic linkages with a molecular weight of 10^7-10^9 (Hoover 2001; Pérez et al. 2009; Waigh et al. 1998). Figure 2.1 shows the chemical structures of amylose and amylopectin. Amylose and amylopectin ratios vary depending on the source of material or plant generating the starch. Waxy starches have very low amylose content (0~5%), while pulse starches contain 35~70% of amylose. Amylose content of pulse starches is higher than that in normal potato (21~25%) and cereal starches (20~35%). Table 2.1 gives an overview of amylose and amylopectin ratios depending on the botanical source (Belitz et al., 2009; BeMiller & Whistler, 2009; Fredriksson et al., 1998; Hoover et al., 2010; Perez & Bertoft, 2010).

Table 2.1 Amylose and amylopectin content depending on the starch source

Sources	Amylose Content %	Amylopectin Content %
Waxy Starch	0~5	35~75
Normal Potato	21~25	75~79
Normal Starch	20~30	70~80
Cereal Starch	20~35	65~80
Pulse Starch	35~65	35~65

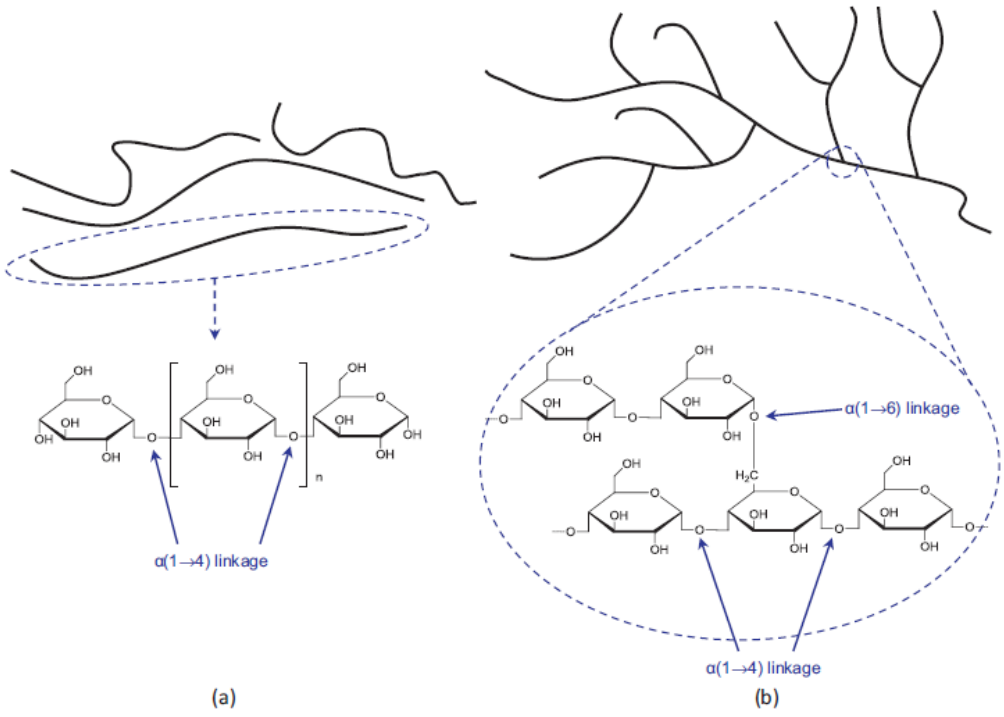

Figure 2.1 Chemical structure of linear amylose (a) and branched amylopectin (b), taken from Xie et al.(2013)

Structurally different amylose and amylopectin molecules form a semi-crystalline aggregate through physical bonding and exist in granular form. In addition, starch particles are formed by repeatedly alternating superposition of a crystalline region and an amorphous region (Pravisani et al., 1985). Amylose has very long chains which can easily form single or double helices (Takeda et al. 1989). On the other hand, amylopectin has a unique branched structure which is often described by a cluster model (Rekha et al. 2006; Thompson 2010a). An amylopectin chain is located within a single cluster or is responsible for linking two or more clusters (Hizukuri 1986).

2.1.2 Gelatinization and Retrogradation of Pulse Starches

In the form of starch natural granules, starch does not dissolve in cold water, whereas when starch is heated in a sufficient amount of water, starch swells irreversibly and loses its crystal structure, which is known as gelatinization. Starch gelatinization is an important phenomenon that occurs in many food processing operations, produced rapidly digestible nutritional content in foods. Differential scanning calorimetry (DSC) is useful to determine the gelatinization parameters of pulse starches. However, it is difficult to make a clear decision as whether these DSC variables are actually representative of species. It is not possible to discuss the effect of molecular structure on the DSC parameters of pulse starch, since the information on the

distribution of amylose and amylopectin chains is not clear (Hoover et al. 2010).

Starch retrogradation is a process that when starch is heated in the water and cooled, the disrupted amylose and amylopectin chains can be progressively recombined into different alignment structures in a process called retrogradation (Wang et al. 2015). Changes in starch during gelatinization and retrogradation are key factors of functional properties for food processing and industrial applications (Wang et al. 2013).

2.2 Resistant Starches

It has been reported that consumers are increasingly demanding low-calorie foods as obesity, diabetes, arteriosclerosis and heart disease are increasing in the world (Tie et al. 2007; Mun & Shin 2002). Dietary fiber has diverse effects and exhibits a variety of physiological characteristics, making it a favorite food material for low-calorie foods. However, foods containing or supplemented with dietary fiber have a characteristic odor and rough texture, which not only deteriorates the quality of the food, but also has a high water absorption capacity, which may result in deterioration of the food during storage (Choi et al. 2005). On the other hand, resistant starch (RS), which exhibits physiological characteristics similar to dietary fiber, is soft without any odor, has a small particle size and exhibits a low water absorption capacity. Therefore, when RS is added to processed foods such as cereal or

snacks, it could maintain a texture of food better than dietary fiber that has been used so far as low calorie materials. It is also possible to develop dietary food products, and it is said to be a food material that can be used in a wide range of foods (Alexander 1995; Huang 1995; Thompson 2010b).

RS has been known since the 1980s, and has become recognized as a dietary fiber-like material; the definition of dietary fiber has included resistant starch since 2001 (Englyst et al., 1982; IOM 2001). Englyst et al. (1992) defined RS as the starch fraction not hydrolyzed by enzymes after 120 min of incubation. And RS is also defined as a starch that is not hydrolyzed in the small intestine of a healthy human body and its hydrolysate (Rekha et al. 2006). Therefore, these properties prevent colon cancer, and are effective for diabetes and obesity, which is physiologically very useful to modern people (Brouns et al. 2002; Xie & Liu 2004).

In terms of digestibility, starch can be classified into three kinds of dietary starches: (1) rapidly digestible starch (RDS), which is defined as a ‘starch which is rapidly converted to glucose by enzymatic digestion within 20min’. RDS is likely to be digested in the human intestine; (2) slowly digested starch (SDS), which is defined as a ‘starch which is converted to glucose after 120min of enzymatic digestion’. SDS is the fraction of starch that has a long digestion time than RDS, but is still digested in the small intestine; and (3) resistant starch (RS), which is

defined as a ‘starch which is not hydrolyzed to glucose in the small intestine within 120min.. RS is the portion of starch has resistant digestion in the small intestine of healthy human but is fermented in the colon’. Table 2.2 indicates the classification of starch and their explanations (Englyst et al. 1992; Pinky 2015).

Table 2.2 Classification of starch, from Pinky et al. (2015)

	Starch fractions		
Item	RDS	SDS	RS (types 1–5)
Digestion timeline (*in vitro*)/place	Within 20 min/mouth and small intestine	20–120 min/small intestine	>120 min/not in small intestine, main action in colon
Examples	Freshly cooked food	Native waxy maize starch, millet, legumes	Raw potato, staled bread
Amount (g per 100 g dry matter)	Boiled hot potato: 65	Boiled millet: 28	Raw potato starch: 75
Main physiological property	Rapid source of energy	Slow and sustained source of energy and sustained blood glucose	Effects on gut health (e.g. prebiotic, fermentation to butyrate with hypothesized anticarcinogenic effects)
Structure	Mainly amorphous	Amorphous/crystalline	Dependent on type, mainly crystalline

RS is classified into 5 major types. Table 2.3 shows some specific features of each RS type (Englyst et al. 1992; Fuentes-Zaragoza et al. 2011; Pinky et al. 2015, Sharma et al. 2008). RS1 is a starch that is physically trapped as partially milled grains or seeds and is difficult to access with enzyme. RS2 is a starch that has a B-crystalline form, such as undamaged starch and high amylose starch, which is separated from fresh potato, banana and beans. RS3 is a retrograded starch (or amylose crystals) that is aged by cooling after the starch is gelatinized. RS4 refers to chemically modified starches to obtain resistance to enzymatic digestion. And RS5 means a starch is characterized by a

lipid that has complexed with amylose to form a helical structure that contains a fatty acid within the central cavity (Dupis et al. 2014; Englyst et al. 1992; Erlingen et al., 1993; Nugent, 2005; Thompson, 2000b).

Table. 2.3 Classification of types of RS

RS type	Description	Food sources	Resistance minimized by	Digestion in small intestine
RS1	Physically protected	Whole or partly milled grains and seeds, legumes	Milling, chewing	Slow rate; partial degree; totally digested if properly milled
RS2	Ungelatinized resistant granules with type B crystallinity, slowly hydrolysed by α-amylase	Raw potatoes, green bananas, some legumes, high-amylose corn	Food processing and cooking	Very slow rate; little degree; totally digested when freshly cooked
RS3	Retrograded starch	Cooked and cooled potatoes, bread, cornflakes, food products with repeated moist heat treatment	Processing conditions	Slow rate; partial degree; reversible digestion; digestibility improved by reheating
RS4	Chemically modified starches due to cross-linking with chemical reagents	Foods in which modified starches have been used (e.g. breads, cakes)	Less susceptible to digestibility *in vitro*	Result of chemical modification; can resist hydrolysis
RS5	Amylose–lipid complexes	Foods with high amylose content	Not susceptible to hydrolysis by α-amylase	Can resist digestion

There are several *in vitro* methods to determine RS content to imitate the starch digestion in humans (AACC, 2000; Akerberg et al., 1998; AOAC, 2000, 2002; Champ et al., 1999, 2003; Englyst et al., 1992; Goni et al. 1996; McCleary & Monaghan, 2002; Muir & O'dea, 1992, 1993; Prosky et al., 1985; Shin et al., 2004). Some popular methods for RS content are listed in Table 2.4 (Dupis et al. 2014). The proposed methods use differing enzymes, pH of incubation, and digestion time/temperature during the reactions. Therefore, RS results reported in the literature vary as the *in vitro* RS value for the food varies depending on the measurement methods due to these different techniques. While each method has its own advantages and

disadvantages, The Englyst method is the most popular method to measure *in vitro* digestibility.

Table 2.4 Methods for measuring RS

Method for measuring RS	Enzyme treatment	Total digestion time	Sample weight	RS determined by
AOAC 991.43: Total dietary fiber[a]	-Bacterial heat stable α-amylase for 30 min and 98 to 100 °C -Bacterial protease for 30 min at 60 °C -Fungal amyloglucosidase for 30 min at 60 °C	90 min	1.000 ± 0.005 g	Weight difference between the hydrolyzed and the original sample
Megazyme RS[b]/AOAC 2002.02/AACC 32-40	-Pancreatin/ amyloglucosidase solution for 16 h at 37 °C	16 h	100 ± 5 mg	Glucose content of the remaining nonhydrolyzed fraction; hydrolysis completed by 2 M KOH and glucose content determined spectrophotometrically
Englyst method[c]	-Pepsin for 30 min at 37 °C -Pancreatin/ amyloglucosidase/ invertase solution for 120 min at 37 °C	150 min	700 to 900 mg dry weight	Glucose liberated in 120 min subtracted from glucose in the total starch content of the sample
Pancreatin–gravimetric method[d]	-Pancreatin/promozyme (pullulanase) solution for 16 h at 37 °C	16 h	1.0 g	Weight difference between the hydrolyzed and the original sample
Goñi method[e]	-Pepsin for 60 min at 40 °C -Pancreatin for 16 h at 37 °C	17 h	100 mg	Glucose content of the remaining non hydrolyzed fraction; hydrolysis completed by 2 M KOH and glucose content determined spectrophotometrically
In vivo	-Various endogenous carbohydrase, lipases, and protease enzymes	1.6 to 2.4 d[f]	Variable	Using ileostomy patients: Digesta is collected as it exits the ileum and a total starch assay is performed using KOH to hydrolyze the remaining starch with the glucose content determined spectrophotometrically

(1992) method is one of the most commonly used *in vitro* digestibility measurement for starchy foods because of its accuracy (Dupuis et al., 2014; Englyst et al., 1992). RS is determined as the starch fraction which can be calculated by deducting of RDS and SDS values from the total starch content. RS is included in the dietary fiber definitions of the American Association of Cereal Chemists (AACC) in some countries such as the United States, Japan and Australia. Other methods like the Association of Official Analytical Chemists (AOAC, 2000) method 991.43 and Total dietary Fiber (TDF) are also widely used to evaluate RS as TDF (AOAC, 2000; de Vries, 2004; Prosky et al., 1985).

Englyst et al. (1992) and AACC (2000) methods are used to investigate the RDS, SDS and RS fractions of pulse starches, see Table 2.5. There are differences in hydrolysis time and enzyme source between the two measurement techniques. Due to differences in these two test methods, it is hard to make meaningful comparisons of RS between the two.

Table 2.5 Rapid digestible (RDS), slowly digestible (SDS) and resistant starch (RS) content of various pulse starches according to the different measurement methods (Hoover et al., 2010)

Starch source	Method	Digestible starch (%)		RS%
		RDS	SDS	
Black gram[a]	Englyst	9.5	29.6	60.9
Chickpea[b]	Englyst	10.9–23.5	34.8–45.7	33.5–54.3
Chickpea[c]	AACC	21.5–29.9	45.7–57.7	8.14–18.4
Kidney bean[d]	AACC	11.7	65.7	17.2
Lentil[e]	Englyst	5.2–14.8	29.7–41.5	43.7–65.2
Lentil[f]	AACC	16.0–16.9	58.3–62.2	13.0–13.2
Mung bean[g]	Englyst	9.7	40.0	50.3
Navy bean[h]	Englyst	8.2	32.3	59.4
Navy bean[i]	AACC	12.4	65.8	21.9
Pea (smooth)[j]	AACC	18.2–23.8	53.7–59.0	8.1–12.6
Pea (smooth)[k]	Englyst	8.1–19.2	33.9–40.2	40.5–58.0
Pigeon pea[l]	Englyst	4.2	16.9	78.9

2.2.1 Heat and Enzyme Treatment of Starch

There are several physical treatments to increase RS content such as heat-moisture treatment (HMT), annealing (ANN), and high hydrostatic pressure processing, and extrusion. These physical modification methods can be safely used as a modification process

compared to other modification approaches. HMT is a physical modification technologies of starch through treatment of starch granules at low moisture content (< 35% moisture) for a specific time (15 minutes to 24 hours) and heating at between glass transition temperature and the gelatinization temperature (Hoover & Vasanthan 1994a). Similar to HMT, ANN method also heats the starch to a temperature between the glass transition temperature and the onset temperature of gelatinization, but uses a high moisture content (> 40% moisture) for an extended time (24 hours or more) (Hoover & Vasanthan 1994b; Tester & Debon 2000). Thus, moisture content is also an important factor affecting the formation of RS in both HMT and ANN. HMT and ANN methods resulted in an increase of RS content by the amylose and amylopectin fractions becoming rubber state and forming double helices to increase the over stability of the starch granules (Chung et al. 2009).

Enzyme treatment is another method used to increase RS content. This method can increase RS content by increasing the apparent level of amylose mainly through the debranching of amylopectin molecules using debranching enzyme. After retrogradation, this causes amylose to form a tightly packed crystal structure (Dupuis et al. 2014). The result is an increase in overall crystallinity in the starch, which makes it difficult for the enzyme to hydrolyze (Li et al. 2011). There have been studies to increase RS content using debranching

enzymes such as pullulanase, α-amylase, alcalase, and protease (Lee et al. 2010; Periago et al. 1998, Rosell et al. 2012). Among them, many studies on RS have been carried out using pullulanase (Lee et al. 2010; .Jin & Zhang 2011; Perry et al. 2009). Pullulanase hydrolyzes α-1, 6-glucosidic bonds in starch (especially in amylopectin), pullulan, and other polysaccharide polymers (Chang & Lin 2006; Jin & Zhang 2011). The increased degree of debranching of amylopectin will provide chains more opportunity to align or aggregate to form a more crystalline structure like amylose (Guraya et al. 2001). Therefore, this crystalline structure is helpful in RS formation. Berry (1986) reported that when the potato amylopectin was debranching with pullulanase, the RS3 content was significantly increased by the increase of the linear starch chains.

2.2.2 Extrusion method of Starch

Extrusion is an important and popular method in the food industry. Extrusion cooking involves high pressure, high temperature, and high shear, which provides some changes in physicochemical and functional properties of the starch (Riha et al. 1996). Feeding rate, feed moisture content, screw configuration, screw speed, operation temperature, and time are all factors that can affect the properties of starch (Brennan et al. 2011; Singh et al. 2007). High product quality, low cost, high productivity, specific shapes of product, easy to control the color and texture, and energy savings are some advantages of

extrusion over other processing methods (Faraj et al. 2004; Farouk et al. 2000). The mechanism by which extrusion increases RS content produces a chain that is more likely to reverse back to RS3 through higher shear conditions due to depolymerisation of starch molecules (Augustiniano-Osornio et al. 2005). Although sometimes RS content has been reported to decrease after extrusion (Gonzalez-Soto et al. 2007), there are still a lot of studies about the effects of extrusion on RS content with various types of starch (Kim & Lee, 1998; Shin et al., 2002; Unlu & Faller, 1998). Faraj et al. (2004) has investigated that the storage conditions of the extrudates can also affect RS content. At 4 ^{o}C for 24 hours of storage condition before oven drying, RS3 content was slightly increased. Sarawong et al. (2014) used different die temperature, feed moisture content and storage conditions to investigate RS formation with banana flour.

Alternatively, extrusion has been successfully demonstrated for the bulk modification of starch as well, offering a shorter reaction time and continuous production capacity than comparable solution reaction processes. The reactive extrusion process of starch involves a combination of physical and chemical modifications wherein the starch is heated, it was conveyed and mixed towards the die by a single or twin-screw, and formed a solid state (Bertolini, 2010; Moad, 2011; Xie et al. 2006). Single-screw extruder (SSE) and twin-screw extruder (TSE) are most widely used in reactive extrusion. TSE can be classified as

co-rotating or counter-rotating depending on the direction of rotation of the screw, and can be categorized as intermeshing or non-intermeshing screw. Co-rotating intermeshing twin-screw extruders are commonly used in many reactive extrusion processes. This is because of the excellent control over mixing and residence time distribution (RTD), ease of reagent addition and removal of by-products and good heat transfer performances of co-rotating intermeshing twin-screw extruder (White et al. 1987; Xie et al. 2006). Although many studies have been done on extrusion to make RS, it is difficult to compare each other's results because of the differences in shear stresses due to differences in the extruder used (i.e. single-screw or twin-screw), screw configuration and processing conditions (i.e. feed rate, temperature, screw RPM). It is clear that moisture content and various processing conditions affect RS content. However, in this thesis, only one processing condition and fixed amount of moisture content were studied because of time constraints.

2.2.3 Chemical Modification of Starch

To date, the most closely related resistant starch is chemically modified starch (RS4) for the purpose of delaying digestion in the small intestine (Mun & Shin, 2000). RS1, RS2, and RS3 delay the action of starch hydrolytic enzymes in the absence of retrogradation; however, after food manufacturing process with high heat, high pressure, and high shear force, the particle structure and physical bonding structure

of starch are destroyed and the nutritional RS content disappears. On the other hands, in the case of RS4 (chemically modified starch), since the substituted functional groups hindering enzymatic attack still remain in the starch molecules after food manufacturing, they can maintain the bonds resistant to digestion or degradation (Björck et al., 1989). There are several chemical treatment methods include cross-linking, esterification, etherification, lintnerization to make RS4. Figure 2.2 shows a variety of chemical modification methods with starch (Tharanathan R. N. 2005). However, the production and use of chemically modified starch is strictly regulates by federal agencies in

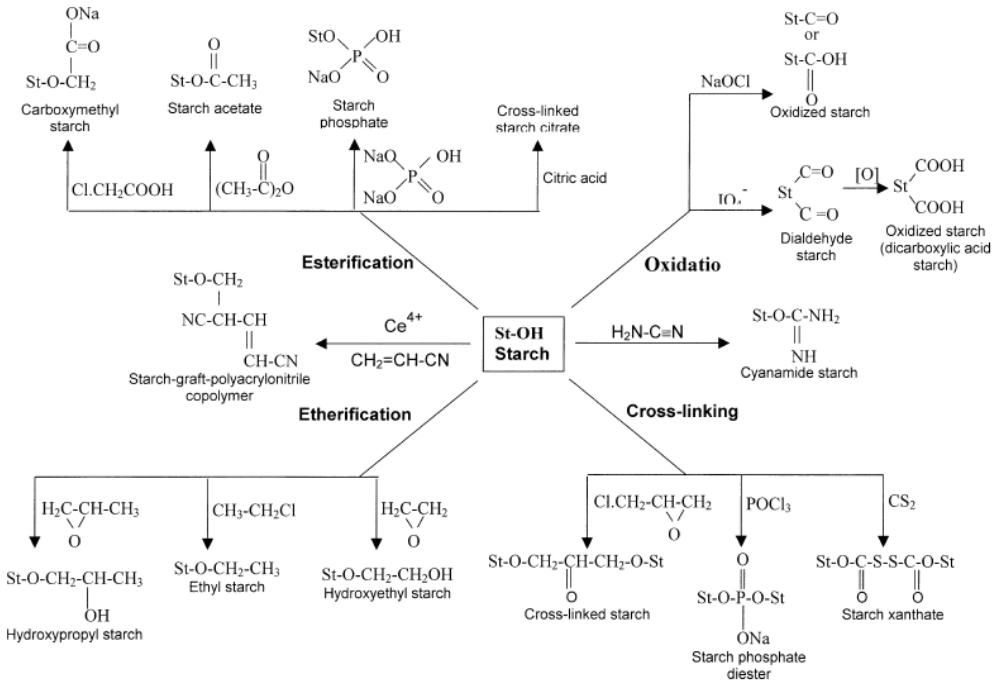

Figure 2.2 Modification reactions of starch (Tharanathan R. N. 2005)

relation to the type of modified starch, the amount of reactants added, the degree of substitution, and the amount of residues remaining

(Dupuis et al., 2014). In addition, until now, consumers' revulsion to chemical synthetic products has increased.

Starch citrate is a modified starch that can minimized the adverse effects of such chemical regulation and repulsion of consumers because citric acid (CA) is a sour ingredient in fruits and is widely used as an acidity regulator and a natural preservative in food application. RS4 can be produced by cross-linking using CA as an additives for chemical modification (Xie & Liu 2004). Reaction scheme of CA with starch is illustrated in Figure 2.3 (Tharanathan R. N. 2005). When CA is heated above 100 ^{o}C, it is dehydrated to form citric anhydride between adjacent carboxyl groups in the same molecule. The anhydride initiates esterification through an additional dehydration reaction with the hydroxyl groups of the starch. Finally, the starch citrate can react with another starch molecule to yield cross-linked starch citrate (Tharanathan R. N. 2005; Xie & Liu 2004).

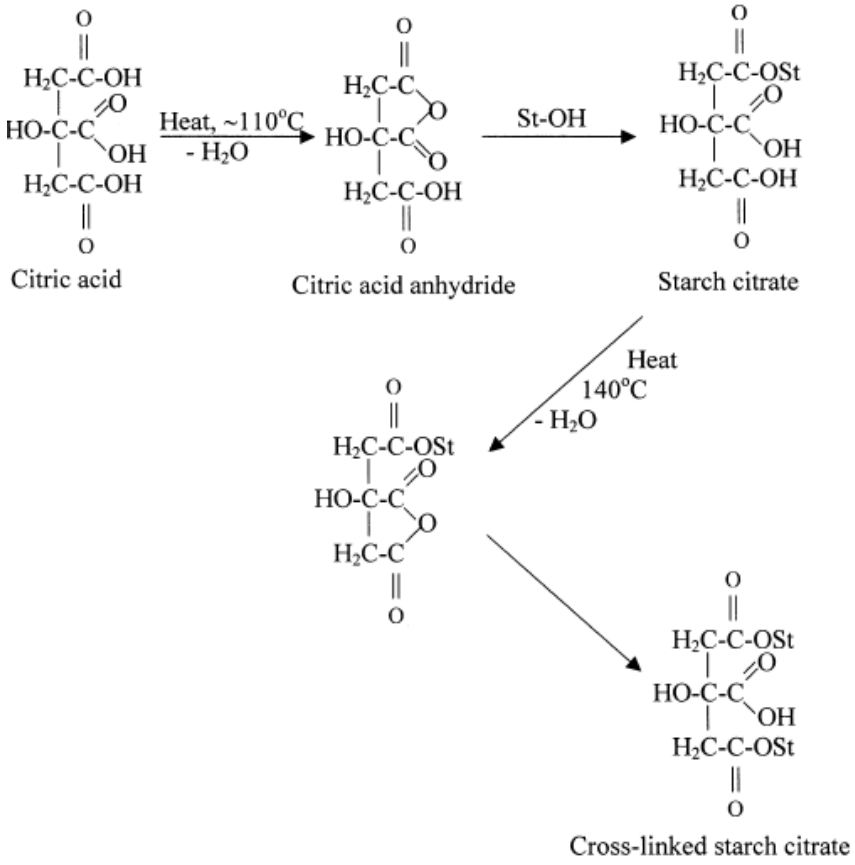

Figure 2.3 Reaction scheme of citric acid and starch

Polycarboxylic acids are considered to be in the category of food safe chemicals for starch cross-linking reactions like with citric acid and have low costs. Malonic acid was used to produce cross-linked potato and corn starch (Blay et al. 2003; Dastidar & Netravali, 2012). Seidel et al. (2001) introduced other polycarboxylic acids such as malic acid, succinic acid, citric acid, and adipic acid to form cross-linked starches. It is known that polycarboxylic acids having two or more carboxylic groups have better crosslinking efficiency than dicarboxylic acid (Yang & Wang, 2000). 1,2,3,4-butanetetracarboxylic acid (BTCA) has four carboxylic groups, while citric acid has three carboxylic groups. It

means that BTCA can provide increased reaction efficiency for esterification versus citric acid. Sodium hypophosphite monohydrate (SHP) has been used to accelerate the esterification reaction and in reality it is used at such high concentrations (50%) that it is difficult to consider it as a catalyst. SHP is also environmentally harmful and an expensive material. Alternatively, sodium propionate (NaP) can replace SHP because it is inexpensive compared to SHP, requires little to be effective, and it is an environmentally friendly catalyst, being also used as a preservative in food application. Scheme of esterification reaction with BTCA and NaP is shown in Figure 2.4 (Netravali & Patil, 2016).

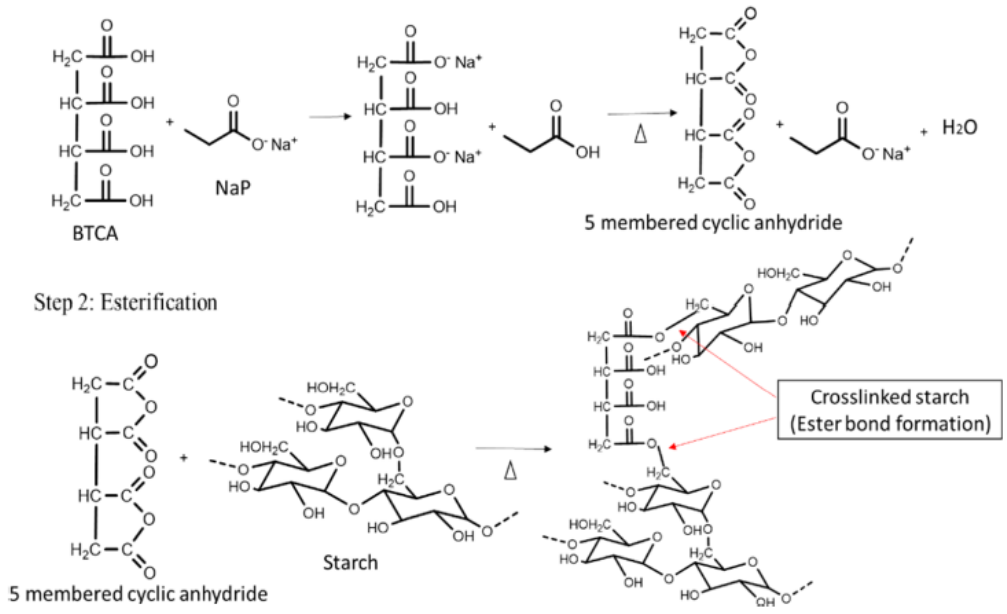

Figure 2.4 Esterification scheme of BTCA as a cross-linking agent and NaP as a catalyst with starch from mango seed, taken from Netravali & Patil (2016)

2.2.4 Epoxidation Modification of Starches

Epoxidized vegetable oils are widely used as plasticizers for polyvinyl chloride, chlorinated rubber and polyvinyl alcohol materials but also act as starting materials for a lot of chemicals, such as alcohols, glycols, carbonyl compounds, olefinic compounds, and polymers due to the high reactivity of their oxirane ring (Liu et al. 2005; Rios et al. 2005; Lin et al. 2008). The most popular epoxidized vegetable oil is epoxidized soybean oil (ESO), though epoxidation has been studied for many long chain olefin and unsaturated fatty acid derivatives of various vegetable oils such as linseed, rapeseed, safflower, olive, corn, melon seed, and cotton seed (Okieimen et al. 2002).

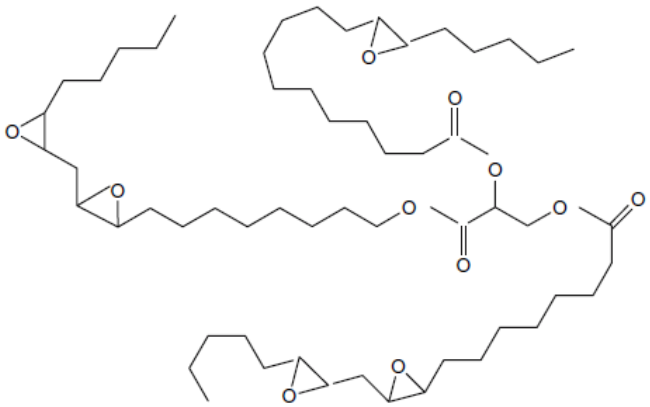

Figure 2.5 Structure of epoxidized soybean oil, taken from Guo et al. (2007)

Generally, there are four methods to produce epoxides from unsaturated olefinic materials (Goud et al. 2011): (1) percarboxylic acid

in the presence of acids or enzymes as catalysts; (2) organic and inorganic peroxides; (3) halohydrins; or (4) molecular oxygen. High conversions are found with percarboxylic acids or halohydrins, though the latter is environmentally unfriendly. Molecular oxygen is the cheapest approach and shows high selectivity, but the activity of this method is low. Thus, the first method with percarboxylic acid is a well-known industrial method.

Only a few studies have investigated epoxidation reactions with canola oil. Canola oil is an adequate replacement to soybean oil in the epoxidation industry because it has about 60% of oleic acid and is a significant product of Canada (Goud et al. 2011). Table 2.6 and Figure 2.6 show the fatty acid compositions and chemical structure of some vegetable oils. Oils heavily composed of oleic acid are less desirable for the purposes of crosslinking in the thesis project since they have only one double bond, whereas those richer in linoleic acid or linolenic acid with two or three double bonds, respectively are more desirable (Maji et al. 2017).

Table 2.6 Typical fatty acids compositions of vegetable oils, taken from Gunstone (1996)

Plant origin	Unsaturated fatty acids**, %**		
	Oleic acid	Linoleic acid	Linolenic acid
Canola oil	56	26	10
Safflower oil	14	75	0
Soybean oil	43	35	3

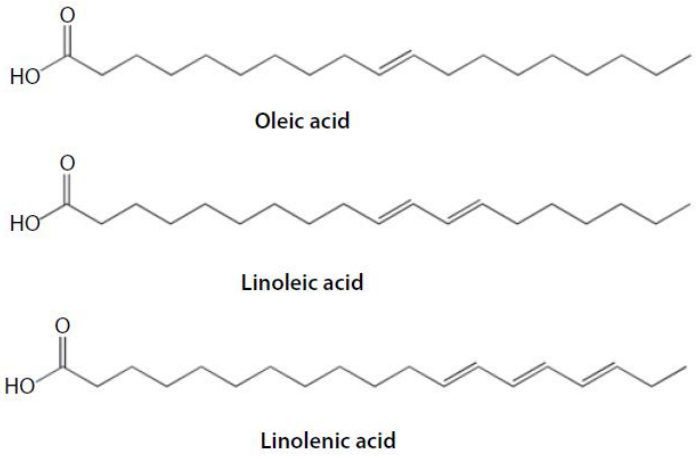

Figure 2.6 Structures of oleic acid, linoleic acid, and linolenic acid, taken from Maji et al. (2017)

CHAPTER 3. EXPERIMENTAL

3.1 Materials

Native pea starch (Accu-Gel^{TM}) was received as donation from Nutri-Pea Limited (Portage la Prairie, MB). This product is a food-grade pea starch which has approximately 12.7% initial moisture content based on moisture analysis (using a Mettler-Toledo HG63). Citric acid (251275, ≥99.5% purity), 1,2,3,4-Butanetetracarboxylic acid (257303, ≥99% purity), sodium propionate (P1880, ≥99.0% purity), potassium hydroxide pellets (C.A.S. No: 1310-58-3), sodium hydroxide pellets (C.A.S. No: 1310-73-2), phenolphthalein (P9750, pH indicator), potassium bromide (FT-IR grade, ≥99.0% trace metals), pullulanase microbial (E2412), carbon tetrachloride solution (48604, 200 μg/mL in methanol), potassium iodide (746428, ACS reagent, ≥99.0% purity), iodine monochloride solution (Wij's solution, 291048, 1.0M in methylene chloride), sodium thiosulphate (725049, ≥98% purity), epoxidized soybean oil (43956, analytical standard) were supplied from Sigma Aldrich (St. Louis, MO). Acetic acid (ACS reagent), sulfuric acid (ACS reagent), hexane (ACS reagent), acetone (ACS reagent) were purchased from CALEDON Laboratory Chemicals (Georgetown, ON). Canola oil (commercial food-grade) was obtained from Metro Inc. (Montreal, QC), safflower oil (commercial food-grade) was obtained from Sobey Inc. (Mississauga, ON). Hydrogen peroxide solution (30 wt% in H_2O, ACS reagent) was obtained from Fisher Scientific (Mississauga,

ON). Ethyl alcohol (95% purity) was purchased from Greenfield Global (Toronto, ON). Deionized water and Milli-Q water were used in this experiments.

3.2 Preparation of Modified Starch Samples

3.2.1 Preparation of Native Starch with/without Heat Moisture Treatment (HMT)

[Gelatinization procedure]

Initially, 4.2 g of acetic acid and 1.4 g of sodium hydroxide were dissolved in 700 ml of deionized (D.I) water to prepare the acetate buffer solution. 70 g of pea starch samples were put in this 700 ml acetate buffer solution. The mixtures were heated in boiling water for 10 min to make pre-gelatinized starch and then samples were placed in an oven set to 120 ^{o}C for 30 min to finish gelatinization. The samples were cooled down to 40, 50, 60, 70, 80 ^{o}C and continuously stirring for 1 hr or 24 hrs. To end the process, the still-moist samples were then dried in an oven at 120 ^{o}C for 30 min. Samples were cooled down to 50 ^{o}C and washed with 300 ml of ethanol to remove excess acetate buffer and finally washed with 4 L of water. The samples were dried in the oven at 45 ^{o}C for 48 hrs and then ground.

[Heat moisture treatment]

Gelatinized starch samples were prepared with 50% (w/w) of D.I water; the D.I water was added to the pea starch and stirred with it for 1

hr. Wet samples were placed in the oven for 24 hrs at 59 ^{o}C, as measured by a thermometer. After that, the samples were cooled down to room temperature and ground for storage and analysis. Table 3.1 indicates all formulations of native pea starch samples with/without heat moisture treatment. Figure 3.1 shows a simplified scheme for native starch with/without HMT and enzyme treated starch with/without HMT.

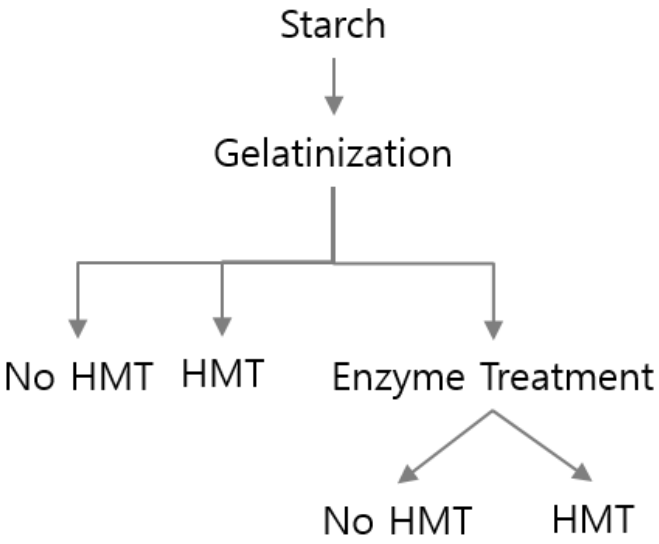

Figure 3.1 Scheme of native starch with/without HMT and enzyme treated starch with/without HMT

Table 3.1 Formulations of Native Pea Starch Samples with/without Heat Moisture Treatment

Sample	Reaction Temperature (℃)	Reaction Time (hr)	HMT	Pullulanase (ml)
NPS-40-1	40	1	N/A	N/A
NPS-40-24	40	24	N/A	N/A
NPS-50-1	50	1	N/A	N/A
NPS-50-24	50	24	N/A	N/A
NPS-60-1	60	1	N/A	N/A
NPS-60-12	60	12	N/A	N/A
NPS-60-24	60	24	N/A	N/A
NPS-70-1	70	1	N/A	N/A
NPS-70-24	70	24	N/A	N/A
NPS-80-1	80	1	N/A	N/A
NPS-80-24	80	24	N/A	N/A
NPS-H-40-1	40	1	O	N/A
NPS-H-40-24	40	24	O	N/A
NPS-H-50-1	50	1	O	N/A
NPS-H-50-24	50	24	O	N/A
NPS-H-60-1	60	1	O	N/A
NPS-H-60-12	60	12	O	N/A
NPS-H-60-24	60	24	O	N/A
NPS-H-70-1	70	1	O	N/A
NPS-H-70-24	70	24	O	N/A
NPS-H-80-1	80	1	O	N/A
NPS-H-80-24	80	24	O	N/A

3.2.2 Preparation of Enzyme Treated Starch with/without Heat Moisture Treatment (HMT)

[Gelatinization procedure & Enzyme treatment]

Acetate buffer was prepared with 4.2 g of acetic acid and 1.4 g of sodium hydroxide were dissolved in 700 ml of deionized (D.I) water. Pea starch (70 g) was put into the 700 ml acetate buffer solution. The mixture was heated in boiling water for 10 min to make pre-gelatinized starch and then the sample was placed in an oven at 120 ^{o}C for 30 min to finish gelatinization. The starch was cooled to 50 ^{o}C and then 3.5 ml of pullulanase was added. Different samples were stirred for 1 hr, 12 hrs or 24 hrs at 40, 50, 60, 70, or 80 ^{o}C. The samples were then dried in an oven at 120 ^{o}C for 30 min to deactivate the residual enzyme. Finally, the samples were cooled down to 50 ^{o}C and exposed 300 ml of ethanol for 30 min to ensure all enzyme was killed. After that the samples were washed with 4 L of water. Finally, the samples were dried in the oven at 45 ^{o}C for 48 hrs before being ground for storage.

[Heat moisture treatment]

D.I water were added to the pea starch to 50% (w/w) and stirred for 1 hr. The samples were placed in an oven at 59 ^{o}C for 24 hrs. After that, the samples were cooled to room temperature and ground for storage. All samples conditions are shown in Table 3.2.

Table 3.2 Formulations of Enzyme Treated Samples with/without Heat Moisture Treatment

Sample	Reaction Temperature (℃)	Reaction Time (hr)	HMT	Pullulanase (ml)
ETS-40-1	40	1	N/A	3.5
ETS-40-24	40	24	N/A	3.5
ETS-50-1	50	1	N/A	3.5
ETS-50-24	50	24	N/A	3.5
ETS-60-1	60	1	N/A	3.5
ETS-60-12	60	12	N/A	3.5
ETS-60-24	60	24	N/A	3.5
ETS-70-1	70	1	N/A	3.5
ETS-70-24	70	24	N/A	3.5
ETS-80-1	80	1	N/A	3.5
ETS-80-24	80	24	N/A	3.5
ETS-H-40-1	40	1	O	3.5
ETS-H-40-24	40	24	O	3.5
ETS-H-50-1	50	1	O	3.5
ETS-H-50-24	50	24	O	3.5
ETS-H-60-1	60	1	O	3.5
ETS-H-60-12	60	12	O	3.5
ETS-H-60-24	60	24	O	3.5
ETS-H-70-1	70	1	O	3.5
ETS-H-70-24	70	24	O	3.5
ETS-H-80-1	80	1	O	3.5
ETS-H-80-24	80	24	O	3.5

3.2.3 Preparation of Acid Modification with Native Starch

[Preparation of acid modification using Haake Mixer]

A Haake mixer (PolyLab Rheomix 3000P with twin roller rotors) was used for bulk modification of starch by cross-linking. Pea starch

(150 g) was dispersed in 150 ml of D.I water with 10 wt%, 20 wt%, 30 wt%, and 40 wt% of citric acid (CA) and stored at room temperature for overnight. The mixture was fed into Haake mixer, heated with all three zones of the device set to 85 ºC. Mixing was conducted at a constant rotor speed of 60 RPM for 6 min. After mixing, the mixture was taken from the mixer and hot-pressed at 85 ºC for 6 min. The pressed sheet (3mm thick) was further allowed to react at different temperatures, 70 ºC, 90 ºC, 110 ºC, 120 ºC, 130 ºC, and 150 ºC for 24hr according to the experimental study in a convective oven; most trials were done at 120 ºC after the initial investigation as discussed in the Results and Discussion chapter. The samples were ground up particles and washed with ethanol to remove unreacted acid species and then washed with water. The cleaned samples were dried at 45℃ for 48 hrs and ground for storage.

[Preparation of acid modification using solution reaction]

Pea starch (20 g) was dispersed in 200 ml of D.I water and heated to 90 ºC for 1 hr. The crosslinking agent, either citric acid (CA) or 1,2,3,4-Butanetetracarboxylic acid (BTCA), was added with/without a catalyst (sodium propionate) and kept at 90 ºC for 1 hr while being stirred at 200 RPM. The mixtures were dried at 120 ºC for 24 hrs in the oven, and then the samples were washed with ethanol to remove unreacted acid species and then washed with water. The cleaned samples were dried at 45℃ for 48 hrs and ground for storage. Figure

3.2 shows a simplified scheme for acid modified starch with CA and BTCA with/without NaP and enzyme/acid treated starch with CA and BTCA with/without NaP.

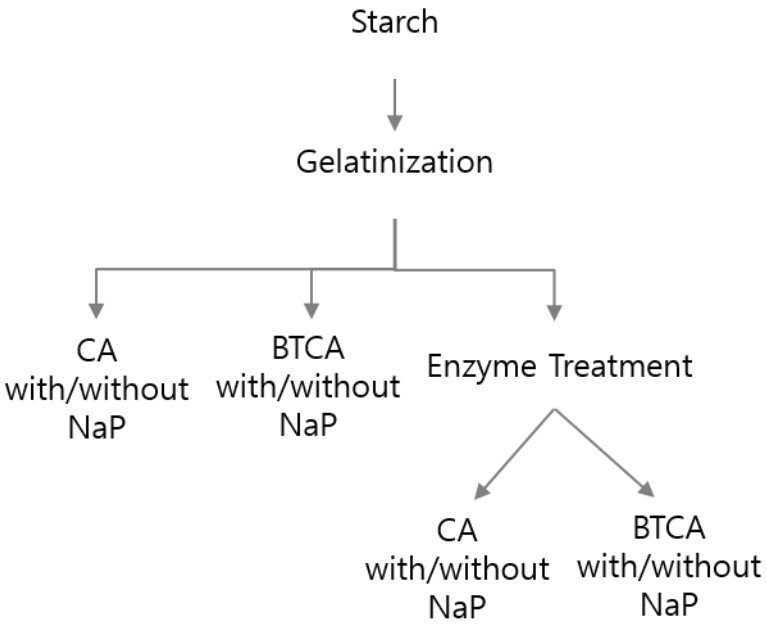

Figure 3.2 Scheme of acid modified starch with CA and BTCA with/without NaP and enzyme/acid treated starch with CA and BTCA with/without NaP

3.2.4 Preparation of Acid Modification with Enzyme Treated Starch

[Enzyme treatment]

Acetic acid (4.2 g) and 1.4 g of sodium hydroxide were dissolved in 700 ml of D.I water to prepare the acetate buffer solution. 70 g of pea starch was dissolved in 700 ml of the acetate buffer solution. The samples were boiled for 10 min and then heated at 120 ^{o}C for 30 min in an oven. The samples were cooled down to 50 ^{o}C and treated with 3.5

ml of pullulanase. The enzyme was allowed to react for 24 hrs at 50 °C before it was deactivated by heating to 120 °C for 30 min. After the reaction, the samples were cooled down to 50 °C and sat in 300 ml of ethanol for 30 min to ensure all enzyme species were deactivated. The final samples were dried at 45 °C for 48 hrs and then ground for acid modification.

[Acid modification]

Enzyme treated pea starch (20 g) was dispersed in 200 ml of D.I water and heated at 90 °C for 1 hour. Crosslinking agent, citric acid (CA) or 1,2,3,4-Butanetetracarboxylic acid (BTCA) at 10 wt% was added with/without 2.5 wt% catalyst (sodium propionate) and kept at 90 °C for 1 hr under constant stirring. Mixtures were dried at 120 °C for 24 hrs in the oven and then washed with ethanol to remove unreacted acid species before finally being rinsed with water. The samples were dried at 45 °C for 48 hrs and ground for storage. All sample formulations are shown in Table 3.3.

Table 3.3 Acid modified samples with/without Pullulanase

Sample	Crosslinking Agent(CA)	CA Amount (%)	Catalyst	Reaction Temperature (°C)	Pullulanase (ml)
NA0-120	N/A	0	N/A	120	N/A
CA10-70	Citric acid	10	N/A	70	N/A
CA10-90	Citric acid	10	N/A	90	N/A
CA10-110	Citric acid	10	N/A	110	N/A
CA10-120	Citric acid	10	N/A	120	N/A
CA10-130	Citric acid	10	N/A	130	N/A
CA10-150	Citric acid	10	N/A	150	N/A
CA20-120	Citric acid	20	N/A	120	N/A
CA30-120	Citric acid	30	N/A	120	N/A
CA40-120	Citric acid	40	N/A	120	N/A
CA10S-120	Citric acid	10	Sodium propionate	120	N/A
BA10-120	BTCA	10	N/A	120	N/A
BA10S-120	BTCA	10	Sodium propionate	120	N/A
NA0-120P	N/A	N/A	N/A	120	3.5
CA10-120P	Citric acid	10	N/A	120	3.5
CA10S-120P	Citric acid	10	Sodium propionate	120	3.5
BA10-120P	BTCA	10	N/A	120	3.5
BA10S-120P	BTCA	10	Sodium propionate	120	3.5

3.2.5 Preparation of Acid Modification Pea Starch Samples Using Twin Screw Extruder

Pre-blended pea starch (1 Kg) was prepared with 10 wt% crosslinking agent in aqueous solution (citric acid or 1,2,3,4-butanetetracarboxilic acid) and 2.5% of catalyst (sodium propionate) in sealed plastic bags. The water content in the pre-blended starch was

30 wt%. The pre-blended samples were kept in a deep-freezer (-40 °C) for 12 hr. After that, bulk esterification of the pre-blended samples was extruded using a Leistritz ZSE-HP 27mm 40L/D co-rotating intermeshing twin-screw extruder (American Leistritz Extrusion Corp.; Sommerville, NJ) without any dies. A high-pressure syringe pump (Teledyne Isco; Lincoln, NE, USA) was used to inject D.I water to reach 80% total moisture (dry starch basis). Table 3.4 showed the formulations for all extrusion samples. A constant temperature profile of 90 °C was applied to all heating zones (9 zones) of the extruder barrel for this study. A constant screw speed of 250 RPM and feed-rate of 3 kg/hr was used. A high-shear screw design was used to compare the degree of cross-linking between a solution reaction method and extrusion. Figure 3.1 shows the screw design which was used in this study. After extrusion, the samples were dried at 120 °C for 24 hrs in an oven, washed with ethanol to remove unreacted acid species and then washed with water. The cleaned samples were dried again at 45 °C for 48 hrs and ground for storage.

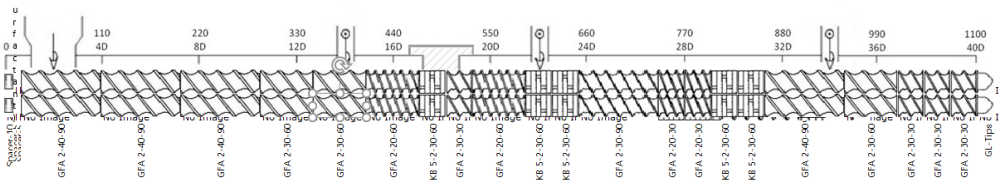

Figure 3.3 Schematic of twin screw design

Table 3.4 Formulations of acid modified samples using twin screw extruder

Sample	Crosslinking Agent(CA)	CA Amount (%)	Catalyst	Total Moisture Content (%)	Extrusion Condition
TSE NPS	N/A	N/A	N/A	80	3.0kg/h, 250RPM
TSE CA	Citric acid	10	Sodium propionate	80	3.0kg/h, 250RPM
TSE BTCA	BTCA	10	Sodium propionate	80	3.0kg/h, 250RPM

3.2.6 Preparation of Epoxidized Pea Starch Samples with Vegetable Oils

[Epoxidation of Canola Oil and Safflower Oil]

The epoxidation procedure reported by Swern (1949) was used with minor modification. A three-neck flask with magnetic stirrer was placed in a water bath kept at 60 °C. Canola or safflower oil (22.6 g) was placed in the flask and 3 g of acetic acid was added as an oxygen carrier to the oil. Sulfuric acid (0.5 g) was used as a catalyst. The mixture was stirred for 30 min. After 30 min, 17 g of 30% (v/v) hydrogen peroxide solution was added drop-wise over 30 min. The epoxidation reaction was allowed to continue for 6, 8, 12, and 24 hrs. The epoxidized oil was washed with water and decanted repeatedly till the pH was near-neutral.

[Preparation of Pea Starch Samples with Epoxidized Oils using Haake Batch Mixer]

A pre-mixture was prepared of 150g of pea starch with 150ml of D.I water before mixing. Mixing was done using a counter-rotating Haake batch mixer (PolyLab Rheomix 3000P with twin Roller Rotors). Mixing temperature and mixing screw speed was kept constant at 85 ^{o}C and 60 RPM, respectively. The pre-mixture was charged into the Haake mixer and 15 ml of either canola oil, safflower oil, epoxidized canola oil, or epoxidized safflower oil was subsequently added. After 6 min of mixing time, samples were removed and taken to a hot press for compression molded at 85 ^{o}C for 6 min. The samples were then put into the oven at 120 ^{o}C for 24 hrs to further react. After 24 hrs, the samples were ground and washed with 500 ml of hexane, filtered and then washed with 500 ml of acetone. The washed samples were dried at 45 ^{o}C for 48 hrs and ground for storage. Figure 3.2 and Table 3.5 show the reaction scheme and formulations for epoxidized samples with canola oil and safflower oil.

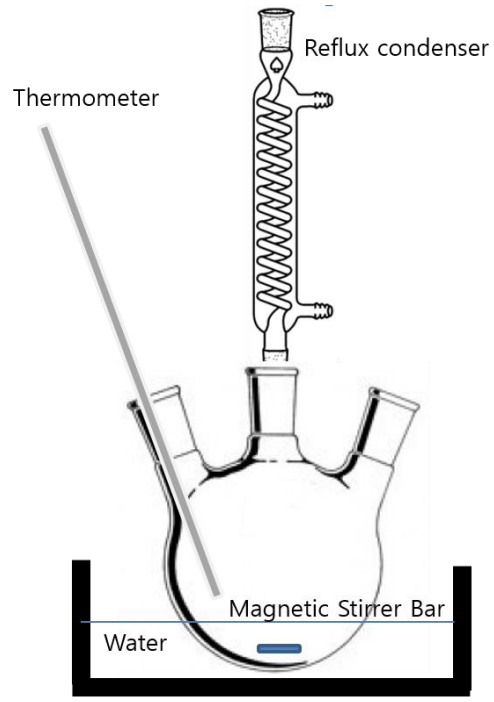

Figure 3.4 Schematic of epoxidation reaction system for vegetable oils

Table 3.5 Formulations of epoxidized samples with vegetable oils

Sample	Vegetable Oils	Epoxidation Conditions		
		Reaction Time (hr)	Reaction Temperature (°C)	Catalyst
EC0	Canola Oil	N/A	N/A	N/A
EC6	Canola Oil	6	60	Sulfuric acid 2 wt%
EC8	Canola Oil	8	60	Sulfuric acid 2 wt%
EC12	Canola Oil	12	60	Sulfuric acid 2 wt%
EC24	Canola Oil	24	60	Sulfuric acid 2 wt%
ES0	Safflower Oil	N/A	N/A	N/A
ES12	Safflower Oil	12	60	Sulfuric acid 2 wt%
ES24	Safflower Oil	24	60	Sulfuric acid 2 wt%

3.3 Characterization

3.3.1 Thermal Properties

Thermal properties of samples were investigated using a Q200 differential scanning calorimeter (DSC; Model Q200, TA Instruments, USA) equipped with a nitrogen purge. All samples were washed with water and/or ethanol and ground prior to testing. The onset (T_o), peak (T_p), and conclusion (T_c) temperatures for gelatinization and the enthalpy of gelatinization (ΔH) were calculated based on the dry weight of the samples. A pea starch sample (4-6 mg) was weighed into a Tzero hermetic pan and scanned from 20 ℃ to 160 ℃, cooled down to 20 ℃ and heated to 160 ℃ again at a rate of 10 ℃ /min. A constant nitrogen flow of 50 ml/min was used for all DSC measurements. The TA Universal Analysis 2000 software was used to analyze the DSC curves and to identify any thermal changes in the pea starch samples.

3.3.2 Fourier Transform Infrared Spectroscopy (FT-IR)

The FT-IR spectra of native and modified starch samples were collected using a ThermoFisher Scientific Nicolet 6700 spectrophotometer (Waltham, MA). Each sample was prepared as a pressed pellet with FT-IR grade potassium bromide (KBr). KBr was dried at 140 ℃ for overnight a day before measuring with the FT-IR. 0.02 g of pea starch was mixed into 0.8 g of KBr powder. The mixture was pressured at 6.89 MPa for 5 min in a die press. The samples were

scanned for the wavenumber range of 800-2000 cm^{-1} with 256 scans at a resolution of 4 cm^{-1}. On the other hand, an ATR method was used to analyze the epoxidized vegetable oil samples to find evidence of epoxidation.

3.3.3 Titration

A colorimetric titration method (AACC 02-01A Fat Acidity General Method, 1999) with minor modification was used to calculate the conversion of acid modified samples. The method was used to estimate the extent of cross-linking. Indicating solution was prepared from 0.1 g of phenolphthalein, 350ml of D.I water and 150ml of ethanol. 1.0 g of acid modified pea starch was prepared in a glass bottle with 50 ml of the indicating solution for overnight. The sample was titrated with 0.089 M KOH until a faint pink colour persisted for 1 min. All samples were titrated in triplicate to increase accuracy. Conversion was calculated based on the moles of acid end-groups.

3.3.4 Water Solubility Index (WSI)

Generally, the water solubility index (WSI) is a common parameter for measuring the degradation of starch, with low values corresponding to less starch being degraded and containing fewer soluble materials (Hernandez et al. (2007). Typical water solubility index values following the traditional methods are too low to compare crosslinking efficiency and so, the procedure reported by Anderson et al. (1969) was used with minor modification. It was modified with KOH

to increase solubility and properly distinguish cross-linked content by ensuring native starch showed a water solubility value close to 100% to make the distinction apparent. A sample (1 g) was dispersed in 25 ml of 1 N KOH in a centrifuge tube. Tubes were put in the oven at 50 °C for 1 hr before centrifuging, to stimulate solubility. Samples were centrifuged at 10,000 g-force for 15 min. After that, 15 ml of the supernatant liquid was poured into a pre-weighted glass beaker carefully and dried at 100 °C overnight. The glass beaker was then weighed to calculate the mass of dried samples in the supernatant. The equation used for water solubility index can be seen below where W_{ss} is the weight of dry solid of supernatant and W_{ds} is the weight of dry sample.

$$\text{WSI} = \frac{W_{ss}}{W_{ds}} \times 100 \quad (1)$$

3.3.5 Cannon-Fenske Viscometer

Intrinsic viscosity was determined according to the Leach method (1963) with some modifications. Cannon-Fenske Viscometers (size 25, 50 and 100) were used to measure the viscosity of 0.1 wt%, 0.2 wt% and 2.0 wt% of starch solution samples in 1 N KOH. Samples were maintained overnight at room temperature to dissolve fully. The viscometer was placed in a water bath controlled to 25 °C. The flow time of the samples were determined manually three times. The Hagenbach correction was provided to calculate the intrinsic viscosity.

For each pea starch concentration, the equations below were used to determine their viscosities.

$$\text{Relative viscosity}, \eta_{rel} = \frac{t}{t_s} \quad (2)$$

$$\text{Specific viscosity}, \eta_{sp} = \eta_{rel} - 1 \quad (3)$$

$$\text{Reduced viscosity}, \eta_{red} = \frac{\eta_{sp}}{c} \quad (4)$$

$$\text{Inherent viscosity}, \eta_{inh} = \ln \eta_{red} \quad (5)$$

where t is the average time of the sample solution, t_s is the average time of the standard solution, and c is the mass concentration of the pea starch sample. Both, the reduced viscosity and the inherent viscosity of the pea starch sample were plotted against concentration of pea starch. The intrinsic viscosity was calculated by extrapolating of reduced viscosity and inherent viscosity to zero concentration of pea starch sample. The final intrinsic viscosity was calculated by averaging of two calculated intercept values.

3.3.6 *In-vitro* Digestibility of Pea Starch Samples

Englyst et al. method (1992) with minor modifications was used to determined rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) content of native and modified pea starches samples. A dried and grounded pea starch sample (100 mg) was mixed with 2 ml of guar gum, 15 glass beads in 4 ml of sodium

acetate buffer solution (pH 5.2) by a vortex mixer. After 5 min of continuous water bath shaking at 37 ^{o}C, 200 strokes/min, 1 ml of enzyme solution was added to test-tube which was consisted of porcine pancreatic α-amylase (Sigma P7545), amyloglucosidase (Megazyme E-AMGDF) and invertase (Sigma I4504). The sample was incubated in a water bath at 37 ^{o}C with 200 strokes/min of shaking. 100 µl of aliquots of hydrolyzed solution was taken at different time intervals of 20 min and 120 min. 1 ml of 50% ethanol was added to terminate the enzymatic reaction. The glucose released in a sample was determined with a glucose oxidase peroxidase diagnostic kit purchased from Megazyme. The absorbance of sample against the reagent at 510 nm of wavelength was measured with a UV/VIS spectrophotometer. The rapidly digestible starch (RDS) was defined as the starch fraction which was hydrolyzed within 20 min of digestion time and was calculate by multiplying glucose (mg) released by 0.9 and divided by 100 mg. With the same procedure, the slowly digestible starch (SDS) was the starch content at 120 min of digestion time minus the starch fraction at 20 min with the same calculation. The resistant starch was defined as total starch content minus the sum of both slowly digestible starch (SDS) and the rapidly digestible starch (RDS). All samples were analyzed in duplicate and all in-vitro digestibility assays analysis were conducted by Elizabeth Donner at Guelph Food Research Centre.

3.3.7 Total Starch Content

Total starch content of acid modified pea starch samples were determined based on AACC method 76.13 (2013). Megazyme total starch kit was used to investigate the total starch content. All experiments were performed by Elizabeth Donner at Guelph Food Research Centre, however, interpreted by author.

3.3.8 Rapid Visco Analysis (RVA)

A Rapid Visco Analyzer RVA4 (Perten Instruments Australia Pty. Ltd., Warriewood, NSW, Australia) was used to determined the pasting properties of pea starch samples according to AACC 76-21.01 (1999). The viscosity was expressed in cP units, where 1 cP is the same with 1 mPa-s^{-1}. Starch (3 g dried and sieved through a 500 ㎛ mesh) was mixed with 25 ml of Milli-Q water in an aluminium bin. The mixed starch sample was maintained at 50 ºC for 1 min, and it was heated from 50 ºC to 95 ºC at a rate of 6 ºC /min and maintained at 95 ºC for 5 min. And it was cooled down to 50 ºC at 6 ºC /min again and kept for 2 min. The paddle speed was set to 960 RPM for the first 10 s, then adjusted to 160 RPM for the rest of the experiment. Each sample was analyzed in duplicate and averaged them. All experiments were performed by Elizabeth Donner at Guelph Food Research Centre.

3.3.9 Iodine Value

There are several methods to measure iodine value; however, in this study, the Wij's solution method was used (PaquotC., 1979). Acetic acid (700 ml) and carbon tetrachloride (300 ml) were prepared in a brown glass bottle. Iodine monochloride (19 g) was added into the brown glass bottle to prepare the Wij's reagent. Vegetable oil (0.2g) was weighed and placed in a ground-necked bottle, to which 15 ml of carbon tetrachloride was added to dissolve the fat. After, 25 ml of Wij's reagent was added to the mixture. The bottle was shaken gently and was placed in the dark for 1 hr. After 1 hr, 20 ml of the potassium iodide solution and 150 ml of D.I water were added along with a starch solution as an indicator. 0.1 N sodium thiosulphate was used to titrate the epoxidized oil samples. The Iodine value was calculated by Equation (6).

$$\text{Iodine Value} = \frac{12.69 \times T \times (V_1 - V_2)}{m} \qquad (6)$$

From the above equation, T is the exact normality of the sodium thiosulphate solution, m is the sample mass, V_1 is the number of ml of the standardized sodium thiosulphate solution used for the blank, V_2 is the number of ml of the standardized sodium thiosulphate solution used for the sample.

CHAPTER 4. RESULTS AND DISCUSSIONS

Pulse starches exhibit a higher RS fraction than other common food starches such as corn or potato. The current pea starch studied in the work had a reported RS fraction of 18% (Huo et al., 2017) and in that same report it was shown to be quite difficult to increase that fraction value by chemical means. To generate significantly high RS values above the native species, multiple alternatives were explored in this thesis with the intent of combining multiple feasible strategies. Most of the sections below cover investigations of these different approaches, looking to optimize the extent of modification. RS testing only done for the most promising methods.

4.1 Heat Moisture Treatment (HMT) of Samples

4.1.1 Native Pea Starch with/without Heat Moisture Treatment (HMT)

HMT was an ideal first approach to study for increasing RS in pea starch for food applications since it avoids the need for chemicals. Intrinsic viscosity was measured for non-chemically modified starch samples with/without HMT. Figure 4.1 shows intrinsic viscosity results of pea starch without HMT and Figure 4.2 show the intrinsic viscosities of pea starch with HMT. Each sample name denoted in the graphs refer to the type of starch, reaction temperature and reaction time used to condition the sample. For example, NPS-40-1 indicates native pea starch (NPS) at 40 ^{o}C of reaction time for 1 hr. Treatment at 80 ^{o}C had

the highest intrinsic viscosity (73.8±0.7 cP without HMT, 144.7±0.5 cP with HMT), though both 70 ºC and 80 ºC at 24 hours of annealing showed significant rises in intrinsic viscosity. The intrinsic viscosity of samples with/without HMT tended to increase only at reaction temperature 70 ºC and 80 ºC (over gelatinization temperature). There was no significant viscosity change at 50 ºC or below, even with different times. For systems of 60 ºC or above (over gelatinization temperature), intrinsic viscosity tended to increase as the conditioning time increased.

The viscosities of heat moisture treated samples were two or three times higher than those never treated, reflecting conformational changes (amylose double helices aggregation) in the polysaccharide during HMT. Heat moisture treatment is an effective process for preparing resistant starch since the chains become less accessible to enzymes in water; however, this conditioning does not survive cooking.

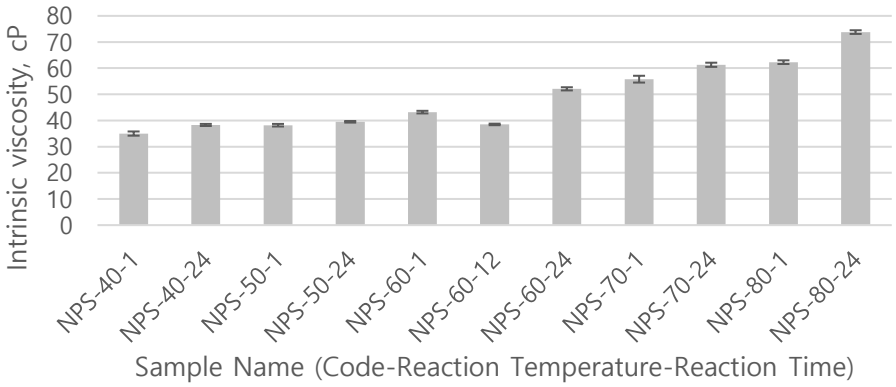

Figure 4.1 Intrinsic viscosity of Non-chemically Modified Pea Starch without Heat Moisture Treatment.

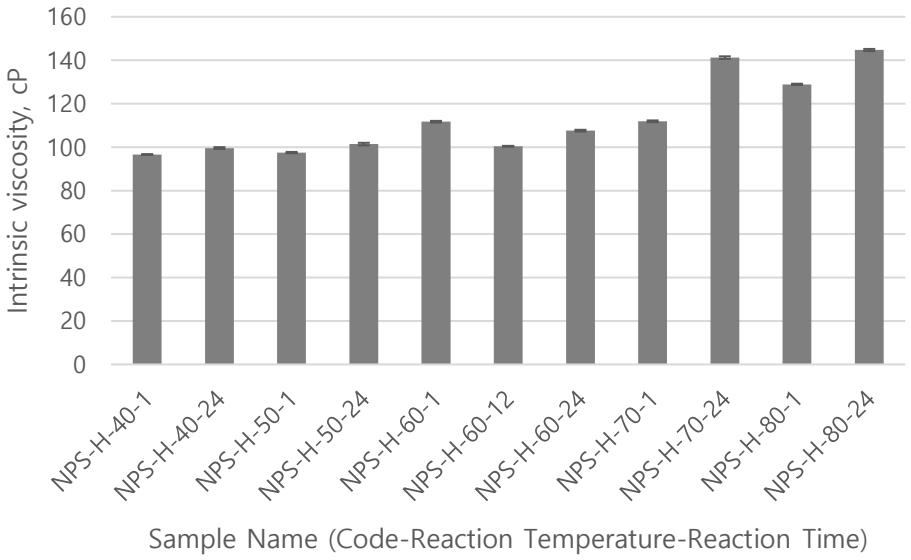

Figure 4.2 Intrinsic viscosity of Non-chemically Modified Pea Starch with Heat Moisture Treatment.

4.1.2 Enzymatic Treatment Samples with/without Heat Moisture Treatment (HMT)

A debranching enzyme (pullulanase) was used to modify the starch prior to HMT to improve the efficiency of preparing resistant starch. The intrinsic viscosities of enzyme treated samples with/without heat moisture treatment are shown in Figure 4.3 and Figure 4.4. Figure 4.3 shows the intrinsic viscosity value of enzyme modified pea starch without HMT whereas Figure 4.4 give them after HMT. Once again, each sample name refers to the type of starch, reaction temperature and reaction time. In this case, ETS-40-1 indicates enzyme treated starch at 40 ºC for a reaction time for 1 hr. An artefact of this approach is that all enzyme treated samples without HMT showed lower

viscosities compare to the native starch. All heat moisture treated samples with pullulanase had higher viscosities than those without HMT, just as before. Samples at 50 °C or below showed lower viscosities and time was not a factor. At the higher temperatures above 80 °C with HMT samples, the influence of time was too small to compare than other temperature conditions, except 60 °C.

The intent of using the enzyme was to allow the starch to more closely pack together during HMT and more effectively resist starch dispersion into water. However, the opposite trend was found to be the case and so HMT was quickly deemed undesirable for study to maximize RS.

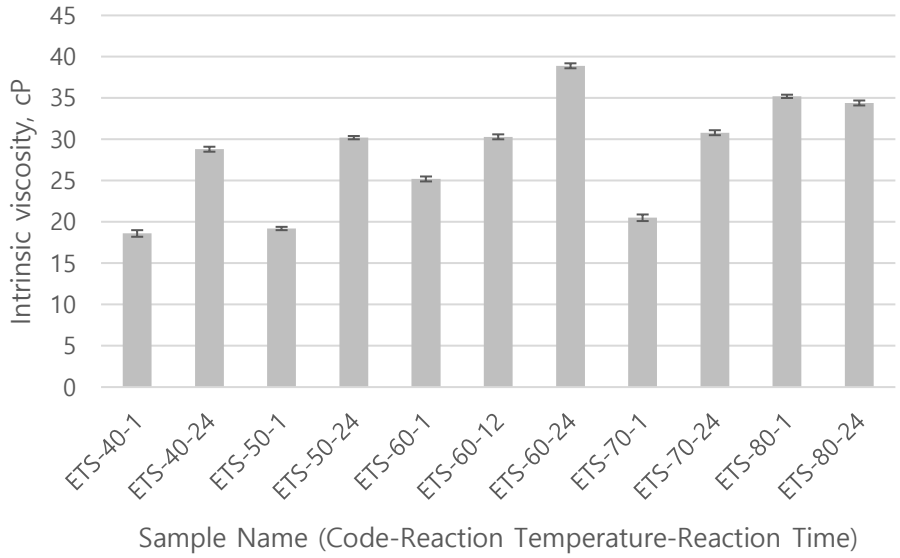

Figure 4.3 Intrinsic viscosity of Enzyme Modified Pea Starch without Heat Moisture Treatment.

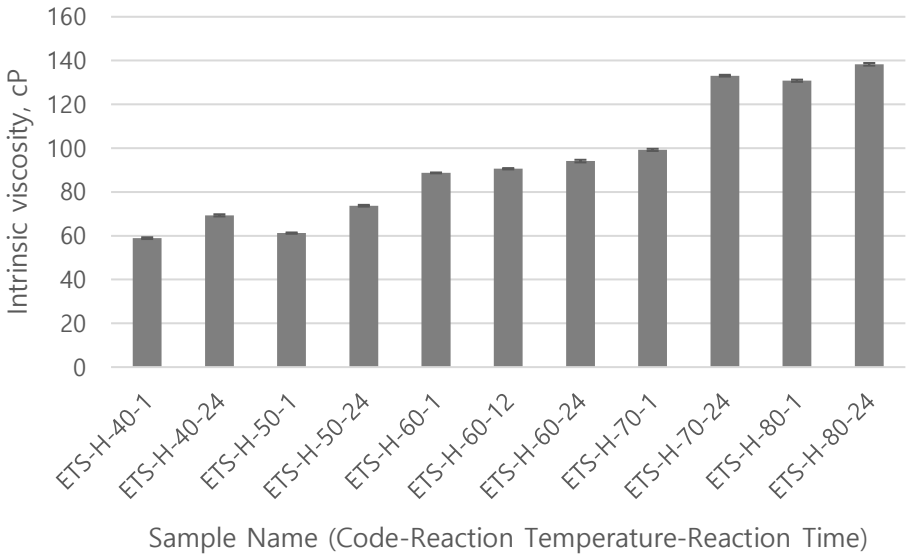

Figure 4.4 Intrinsic viscosity of Enzyme Modified Pea Starch with Heat Moisture Treatment.

4.2 Acid Modified Samples

4.2.1 Citric Acid (CA) modification with Pea Starch

A significant number of experiments were conducted to determine the optimal reaction conditions for crosslinking, initially with the crosslinking agent, citric acid. Since the reaction is reliant upon anhydride formation, the presence of water for gelatinization was an obvious complication to high extents of conversion. WSI and calculated conversion (based on acid group titration) were measured after reaction at different oven temperatures from 70 ºC to 150 ºC with 10% (w/w) citric acid, with results shown in Figure 4.5 and Figure 4.6, respectively.

Figure 4.5 shows the results of water solubility index at the various reaction temperature from 70 °C to 150 °C. Figure 4.6 presents the conversion based on titration for the reaction temperature from 70 °C to 150 °C. Each sample name in the graphs refer to the type of cross-linking agent, amount of cross-linking agent and reaction time. For example, CA10-70 means citric acid 10% at 70 °C. As the temperature increased from 70 °C to 150 °C, the conversion increased, while WSI decreased, showing evidence of more cross-linking with higher temperatures. However, the sample turned slightly yellow due to the heat above 130 °C which would be less desirable for a food ingredient and so, the optimum reaction temperature for cross-linking with 10% citric acid was set at 120 °C with 44.8±1.6 % of WSI and 91.1±2.3 % of conversion. Other studies have shown that citric acid requires a high temperature above 100 °C to form citrate starch (Xie & Liu 2004).

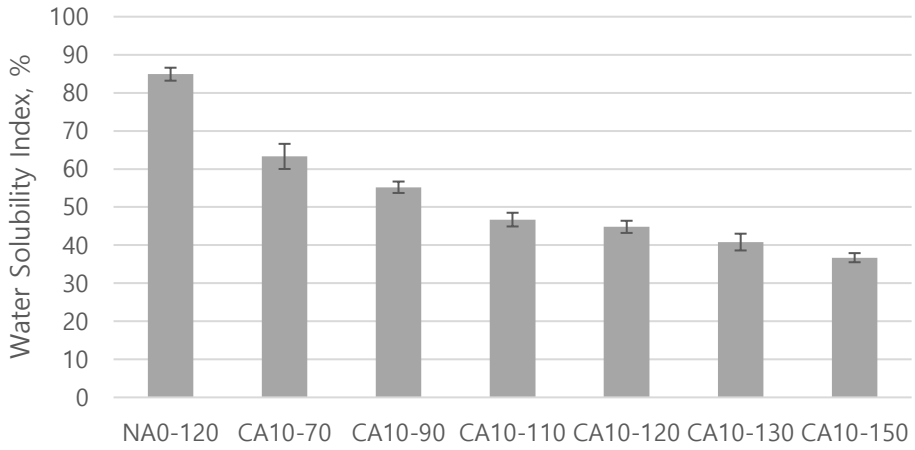

Figure 4.5 Water Solubility Index of Pea Starch Samples at Various Reaction Temperature with 10% Citric acid for 24hr (NA0-120: Gelatinized pea starch only, no citric acid treatment)

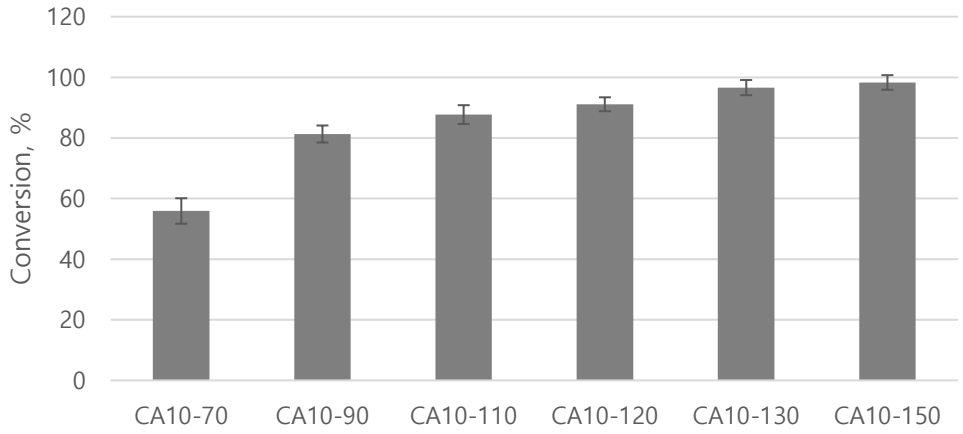

Figure 4.6 Conversion of Pea Starch Samples at Various Reaction Temperature with 10% Citric acid for 24hr

The subsequent trials in this section looking at acid concentration used 120 ºC as the oven reaction temperature. A comparison followed for the effective citric acid concentration in crosslinking the pea starch, as seen in Figure 4.7 and Figure 4.8, respectively. Figure 4.7 shows

the water solubility index of pea starch samples with different concentration of citric acid from 10 % to 40 % at 120 ºC for 24 hr of reaction condition. Figure 4.8 shows the conversion based on titration of the same pea starch samples. The conversion increased from 82.5±2.2 % to 95.1±2.4% as the amount of citric acid increased from 10% to 40%. The WSI was decreased from 48.2±0.8 % to 43.5±0.9 %, respectively, as the amount of citric acid increased from 10% to 40%. This decline of WSI was not large, and showed no difference between the 30% and 40% content of citric acid. Overall, it was decided that a concentration of citric acid above 10% offered insufficient change of WSI and conversion to compensate for the cost of the reactant.

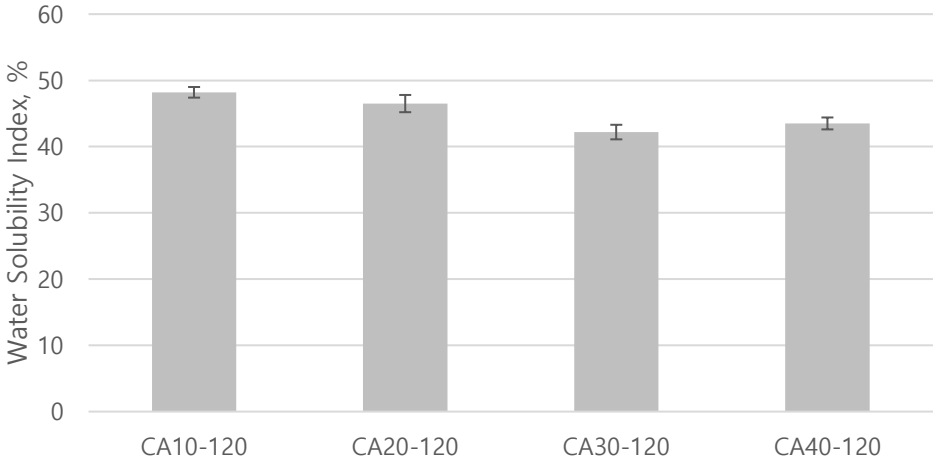

Figure 4.7 Water Solubility Index of Pea Starch Samples at Various Concentration of Citric Acid with 120 ºC Reaction Temperature and Reaction Time of 24hr

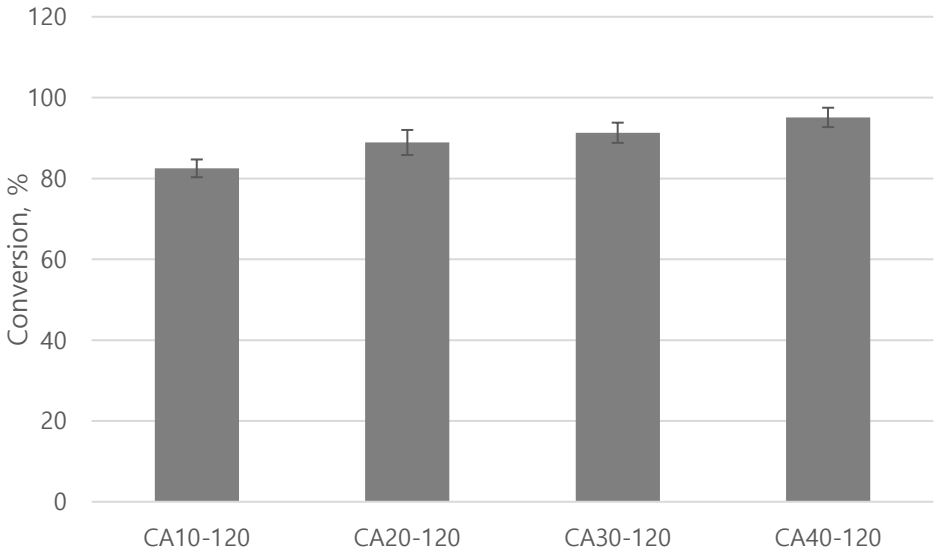

Figure 4.8 Conversion of Pea Starch Samples at Various Concentration of Citric Acid with 120 ºC Reaction Temperature and Reaction Time of 24hr

FT-IR results of starch samples at the various reaction temperatures and different concentrations of citric acid are shown in Figure 4.9 and Figure 4.10, respectively. The broad peak at 1750 cm^{-1} was new in all citric acid treated starch samples, attributed to the formed ester group. Chen et al. (2015) also found a new peak at 1724 cm^{-1} on FT-IR in their citric acid esterification with cassava starch. The peak at 1640 cm^{-1} for native starch was used as a reference. A peak ratio was calculated by dividing the intensity at 1750 cm^{-1} by the intensity at 1640 cm^{-1}. This peak intensity represented that how much citric acid existed in the starch mixture. The peak ratio at 120 ºC reaction temperature reached the highest value as 1.31. After that, the peak intensities were decreased at 130 ºC (1.18) and 150 ºC (1.06),

possibly due to the peak at 1640 cm^{-1} being affected by side reactions which produced the yellowing mentioned earlier. The peak ratio related to the amount of citric acid is included in Figure 4.10. Although the peak ratio of 30% citric acid (1.43) was higher than that of 10 % citric acid (1.31), the difference was not significant.

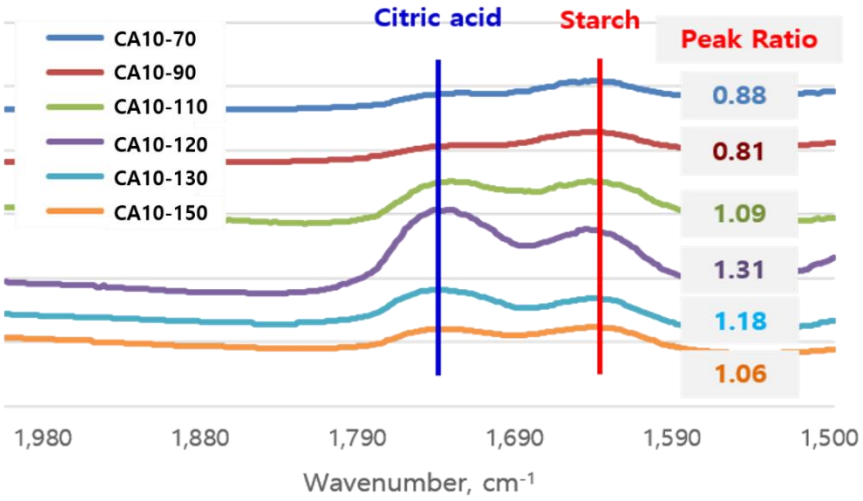

Figure 4.9 ATR FT-IR Spectra of Pea Starch Samples at Various Reaction Temperatures with 10 wt% Citric Acid

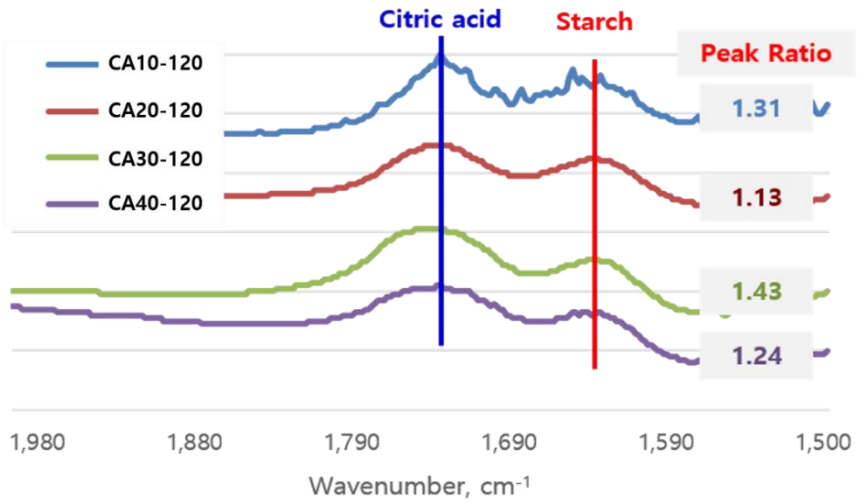

Figure 4.10 FT-IR Spectra of Pea Starch Samples at Various Concentration of 10-40 wt% Citric Acid with 120 ℃

4.2.2 1, 2, 3, 4 – Butanetetracarboxylic Acid (BTCA) modification with Pea Starch

The anhydride group formed by CA at high temperatures is sterically restrained making it less likely to occur, especially under shorter reaction times. An alternative cross-linking agent, 1,2,3,4-butane-tetracarboxylic acid (BTCA) was considered as it would be less sterically restrained and hopefully more successful at producing higher extents of conversion; Netravali & Patil (2016) and Netravali & Dastidar (2013) made cross-linking with starch using BTCA and NaP. BTCA was tested for its performance to improve the conversion and WSI properties, as seen in Figure 4.11 and Figure 4.12; BTCA was compared with CA and now both reactants were examined with/without

a catalyst, NaP. BA10-120 refers to a sample prepared with BTCA (cross-linking agent) at 10% and 120 ^{o}C, while BA10S-120 refers to similar conditions but including 10% sodium propionate (catalyst). The reaction conditions of 120 ^{o}C and a molar equivalent to 10% citric acid were used. BTCA treated starch samples showed generally higher conversion and lower WSI compared to citric acid treated samples. WSI and conversion data with BTCA showed slightly improved crosslinking results over citric acid since its di-anhydride was more accessible to the –OH group of starch for the crosslinking reaction. Similarly, the BTCA case using sodium propionate as a catalyst showed the clearest increase in conversion and decrease in WSI, demonstrating minor effectiveness of the catalyst in the bulk reaction. Sodium propionate does appear beneficial for both citric acid and BTCA, but it was less effective with the former species. For example, the conversion with citric acid was 87.5±3.3 % and citric acid with sodium propionate was 92.6±1.5 %; the conversion with BTCA was 92.1±2.9 % and BTCA with sodium propionate was 96.8±2.3 %. The conversion of BTCA alone showed similar conversion to that of citric acid with sodium propionate. Similarly, WSI of citric acid and citric acid/sodium propionate were 22.9±2.5 % and 17.3±2.1 %, respectively; WSI with BTCA and BTCA with sodium propionate were 20.3±2.3 % and 13.1±2.0 %, respectively.

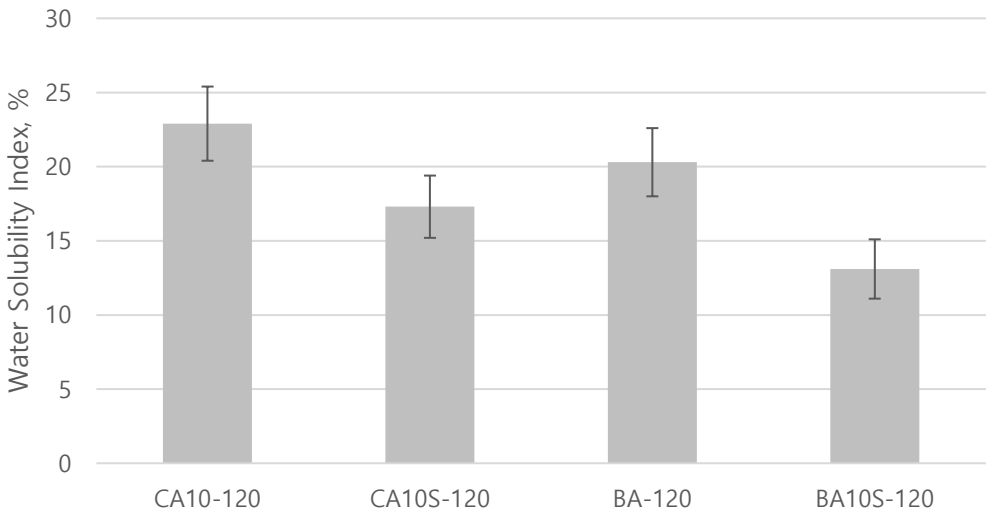

Figure 4.11 Water Solubility Index of Pea Starch Samples with Different Cross-linking Agent with/without Catalyst

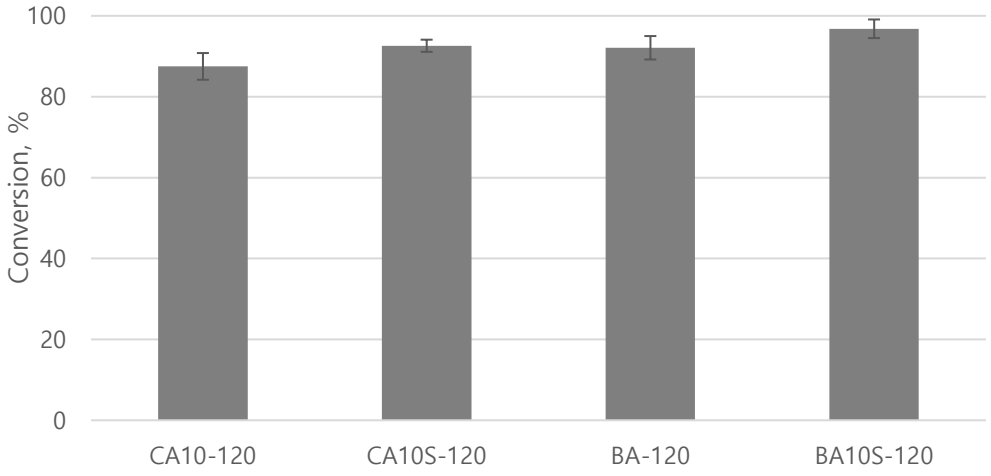

Figure 4.12 Conversion of Pea Starch Samples with Different Cross-linking Agent with/without Catalyst

4.2.3 Acid Modification with Enzyme Treated Pea Starch

The reduced WSI with BTCA (but also CA) was very promising but to investigate how much more crosslinking could occur if the polysaccharide molecules were less constrained, it was decided to examine debranched material. WSI and intrinsic viscosities of enzyme treated starch samples for various modification systems are shown in Figure 4.13 and Figure 4.14, respectively. NA0-120P means pullulanase treated, gelatinized starch prepared at 120 ^{o}C with no cross-linking agent (no acid). CA10-120P refers to a sample made with 10% citric acid (cross-linking agent) for pullulanase treated starch at 120 ^{o}C of reaction temperature. The pullulanase treated samples showed higher WSI than the samples without pullulanase treatment. WSI for the modified debranched starch with cross-linking agents such as citric acid and BTCA indicated a much more dramatic decrease than native species, now dropping from 89.8±2.8 % (NA0-120P) to a range of between 33.6±1.6 % (BA10-120P) and 40.3±1.5 % (CA10-120P). The intrinsic viscosities increased from 11.3±1.4 cP (NA0-120P) to around 26.0±2.7 cP (BA10-120P) and 27.6±2.2 cP (CA10-120P) to denote crosslinking as molecular weight was built back up by the reaction. The WSI of native starch (NA0-120, no pullulanase treatment) was 84.9±1.7 %.

The samples with catalyst, sodium propionates, showed a slightly improved effect on both WSI (CA10S-120P: 38.8±2.2 %, BA10S-120P:

31.7±2.3 %) and intrinsic viscosity (CA10S-120P: 33.3±1.8 cP, BA10S-120P: 29.7±1.5 cP). However, no significant changes were found in the presence of catalyst system compare to pullulanase only treated sample.

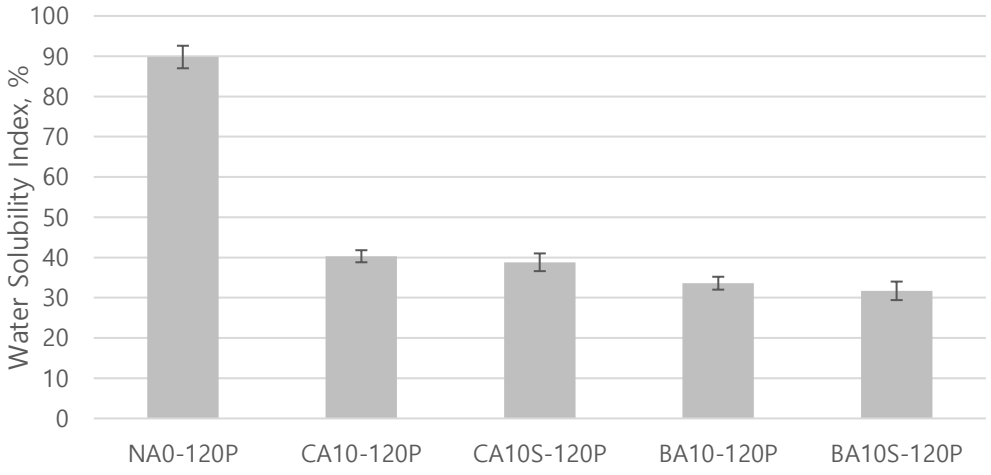

Figure 4.13 Water Solubility Index of Pea Starch Samples at Different Acid and Pullulanase Conditions

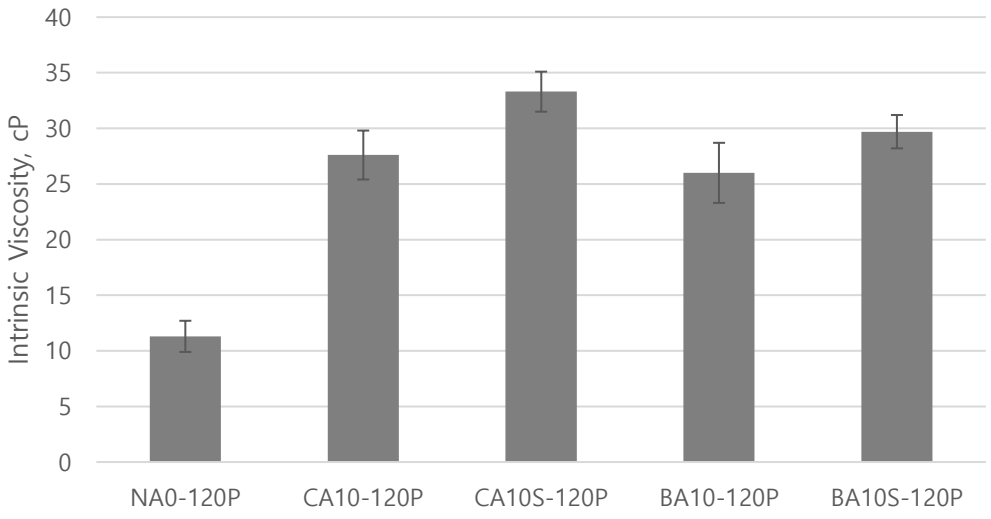

Figure 4.14 Intrinsic Viscosity of Pea Starch Samples at Different Acid and Pullulanase Conditions

4.3 Epoxidized Samples

4.3.1 Measurements of Epoxidized Oils

Crosslinking with either CA and BTCA will be partially inhibited since the distance between their reactive groups requires two polysaccharide molecules to be pulled very close together. To increase the distance between reactive end-group as well as consider less reliance on anhydride reactions, an epoxidized plant oil was investigated. In the absence of commercially accessible epoxidized oils, two types were synthesized internally.

The epoxidation can be confirmed by iodine value, FT-IR, TLC analysis, and NMR (Gamage et al. 2009). Iodine value and FT-IR methods were used to verify the epoxidation of canola oil and safflower oil in this study; canola oil was considered as a product of Canada but it has few chains exhibiting more than one double bond for epoxidation while safflower oil has a much higher frequency of two double bonds per chain meaning that its epoxided species would have a greater potential to crosslink starch. Specifically, canola oil has 92 % of unsaturated fatty acids (56 % of oleic acid, 26 % of linoleic acid, 10 % of linolenic acid) with 6 % of saturated fatty acids, while safflower oil has 89 % of unsaturated fatty acids (14 % of oleic acid, 75 % of linoleic acid) with 10 % of saturated fatty acids (Gunstone 1996). Although safflower has more linoleic acid which has more double bonds, the total amount of fatty acids seems to be more dominant. Table 4.1 shows the

results of iodine value of epoxidized canola oil and epoxidized safflower oil. Iodine value was measured only once due to the limitation of Wij's solution. The iodine values of virgin canola oil and safflower oil were 5.46 and 7.84, respectively. After epoxidation, the iodine values of each oil were lower than that of virgin oils. In the case of canola oil, 12 hr of epoxidation sample showed 59.9% epoxidation, while safflower oil showed little difference (60.0%) but needed a longer epoxidation time (24 hr). Gamage et al. (2009) found some specific processing conditions using rubber seed oil, neem oil, and mee oil with over 88 % of epoxidation.

Table 4.1 Iodine Value of Epoxidized Canola Oil and Epoxidized Safflower Oil

Oils		Results	
		Iodine Value	Epoxidation %
Canola	No epoxidation	5.46	-
	12hr epoxidation	2.19	59.9
	24hr epoxidation	2.82	48.4
Safflower	No epoxidation	7.84	-
	12hr epoxidation	6.07	22.8
	24hr epoxidation	3.14	60.0

Figure 4.15 and Figure 4.16 show FT-IR results of canola oils and safflower oils, respectively. After 24 hr of epoxidation, the peak of epoxy groups (between 822 and 833 cm^{-1}) was observable, as seen in

Figure 4.15. However, there was no peak around at 830 cm^{-1} in 12 hr of epoxidation with canola oil. Petrovic & Vlcek (2006) conducted epoxidation of soybean oil and they also found the same vibrational peak in their research. The peak was obstructed by another in the FT-IR of the safflower oil samples. Although it was seemed that the peak of epoxy group did not appear on FT-IR due to the low epoxidation efficiency, the epoxy group was not observed even under the 24 hr of reaction condition.

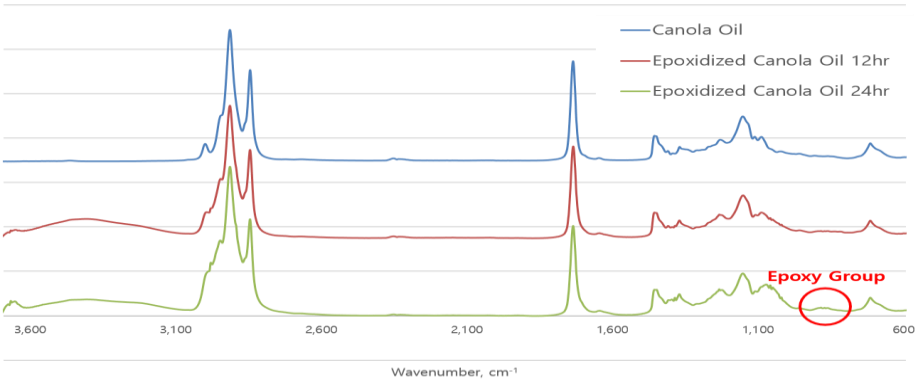

Figure 4.15 FT-IR of Canola Oils at Different Epoxidation Time

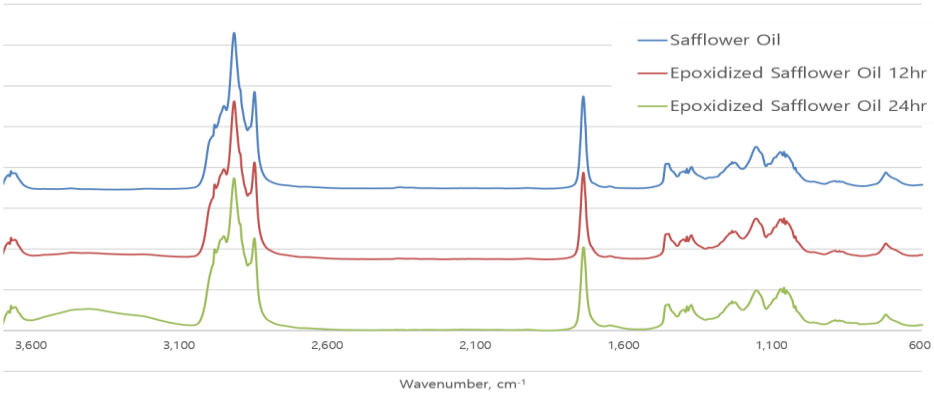

Figure 4.16 FT-IR of Safflower Oils at Different Epoxidation Time

4.3.2 Epoxidation of Pea Starch Samples with Epoxidized Vegetable Oils

WSI of the modified starch samples produced using the epoxidized oils made with vary epoxidation time is shown in Figure 4.17. EC12 refers to epoxidized canola oil, was made from 12 hr of epoxidation, while ES12 corresponds to epoxidized safflower oil with 12 hr of epoxidation. The samples with epoxidized canola oils indicated lower WSI (EC12: 60.7±2.81 %, EC24: 65.9±1.56 %) than the sample with virgin oil (EC0: 71.6±1.25 %). A similar trend was found in the safflower oil samples. However, the samples with virgin oils also showed a fairly low WSI.

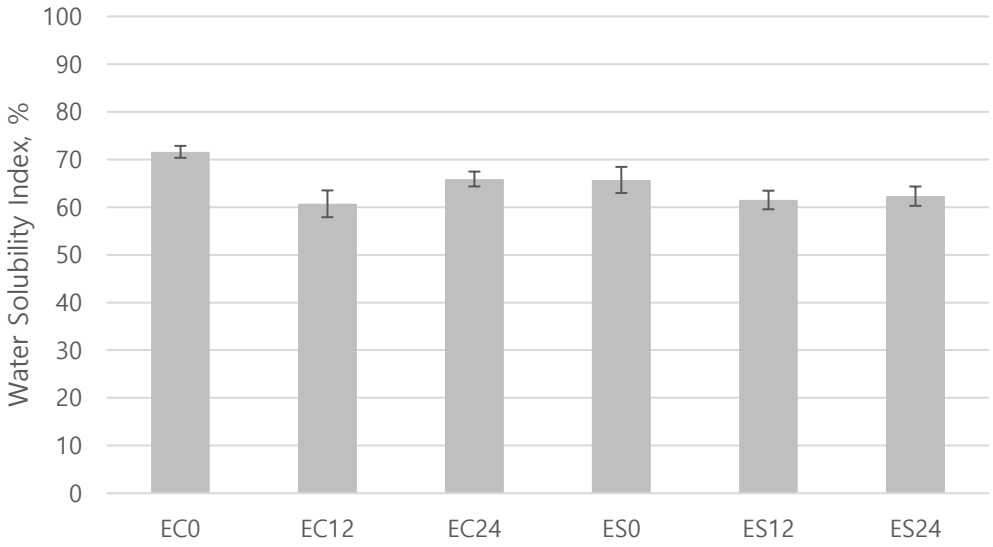

Figure 4.17 Water Solubility Index of Epoxidized Pea Starch Samples

4.4 Extrusion Samples

4.4.1 Acid modification of Pea Starch Using a Twin Screw Extruder

WSI and conversion of acid modified starch samples are shown in Figures 4.18 and 4.19, respectively. BTCA treated sample showed the lowest WSI (22.4±1.11 %) with the highest conversion (87.2±3.08 %). However, the difference between BTCA treated sample and CA treated sample (WSI 28.7±0.92 %, Conversion 83.5±2.87) was small. The results of WSI and conversion for extruded samples were lower than those from the batch mixer (Sec 4.2), but the difference is not large. The difference is due to the shorter residence time for the reaction in the extruder compared to the mixer. A previous study with the similar screw design (Kristi Ciardullo, MASc student) found the mean residence time was 120±10.0 seconds, much less than the 6 minutes in the mixer. Due to time limits in the project, only one processing condition was used in the extruder to produce acid modified starch samples; additional research is needed on the more diverse processing conditions (moisture content, feed rate, screw configuration, and temperature) and the amount of cross-linking agent.

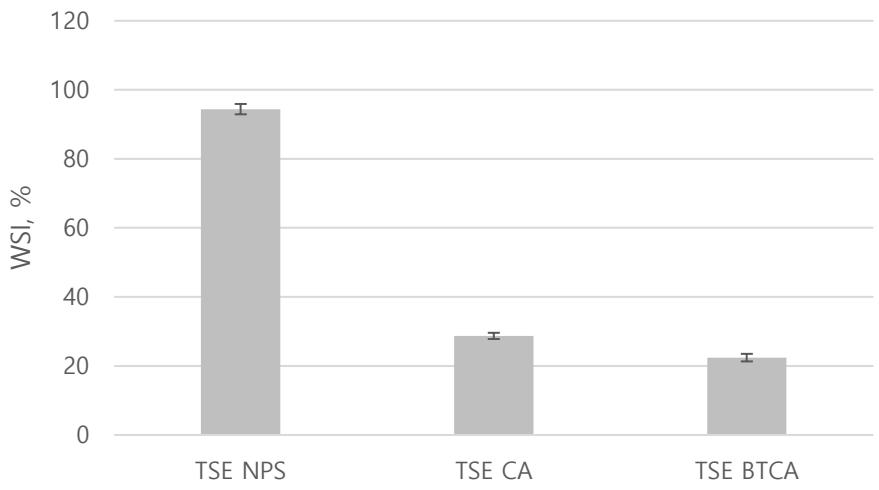

Figure 4.18 Water Solubility Index of Pea Starch Samples using Twin-screw Extruder with Different Cross-linking Agent

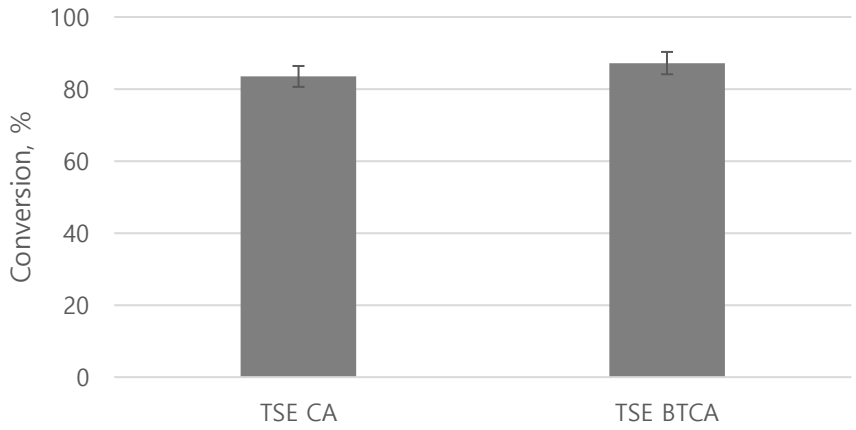

Figure 4.19 Conversion of Pea Starch Samples using Twin-screw Extruder with Different Cross-linking Agent

4.5 In Vitro Digestibility

Various starch samples prepared by different methods in this study were measured by the Englyst method (1992) with minor modifications. Table 4.2 and Figure 4.20 summarizes the nutritional fraction of RDS, SDS, and RS for native and modified pea starch samples. The primary interest of the study was to maximize RS but increasing SDS was also seen as beneficial to the goals of the project. For comparison, the RS and SDS fraction of the native pea starch (NPS) as supplied (i.e. granular) was 18.7 ± 0.6% and 28.8 ± 0.2%, respectively and only 7.8 ± 0.7% and 11.9 ± 1.2%, respectively after gelatinization; the gelatinization values are better comparators in this study since the cross-linked starch species were first gelatinized.

In all cross-linked samples, RS content was higher than native pea starch. Most notable, in the case of the samples modified with the debranch starch species, the fraction of RS was measured to be two or three times higher than without pullulanase. In addition, when using debranched starch, the RDS content was extremely low. BA10S-120P showed the highest RS fraction (80.81±0.18%) produced in the project with the lowest RDS fraction as 13.73±0.26%. For comparison, Zhao & Lin (2009) observed that RS yield increased from 8.5% to 11% for citric acid hydrolysis of retrograded maize starch. They found that citric acid and pullulanase were more effective for increasing RS. In their study, they prepared their samples in a very different manner though, using

an autoclave and cycling the reaction in temperature between 121 °C and 4 °C for 24 hr. In this study, however, since pea starch was debranched by pullulanase prior to cross-linking, degraded starch components were more easily coupled with cross-linking agents.

Table 4.2 Digestion Profiles of Native and Cross-linked Pea Starch

Samples	Cross-linking agent	Pullulanase (ml)	Digestion Profile (Englyst method)		
			RDS (%)	SDS (%)	RS (%)
NPS[A)]	N/A	N/A	52.49±0.39	28.84±0.21	18.67±0.60
GNPS[B)]	N/A	N/A	80.30±0.80	11.90±1.20	7.80±0.70
CA10S-120	CA	N/A	72.95±0.37	4.95±0.67	22.11±0.41
BA10S-120	BTCA	N/A	37.76±2.34	29.90±0.32	32.35±2.06
CA10S-120P	CA	3.5	22.08±1.41	7.80±0.40	70.12±1.79
BA10S-120P	BTCA	3.5	13.73±0.26	5.47±0.20	80.81±0.18

A) Native Pea Starch

B) Gelatinized Native Pea Starch

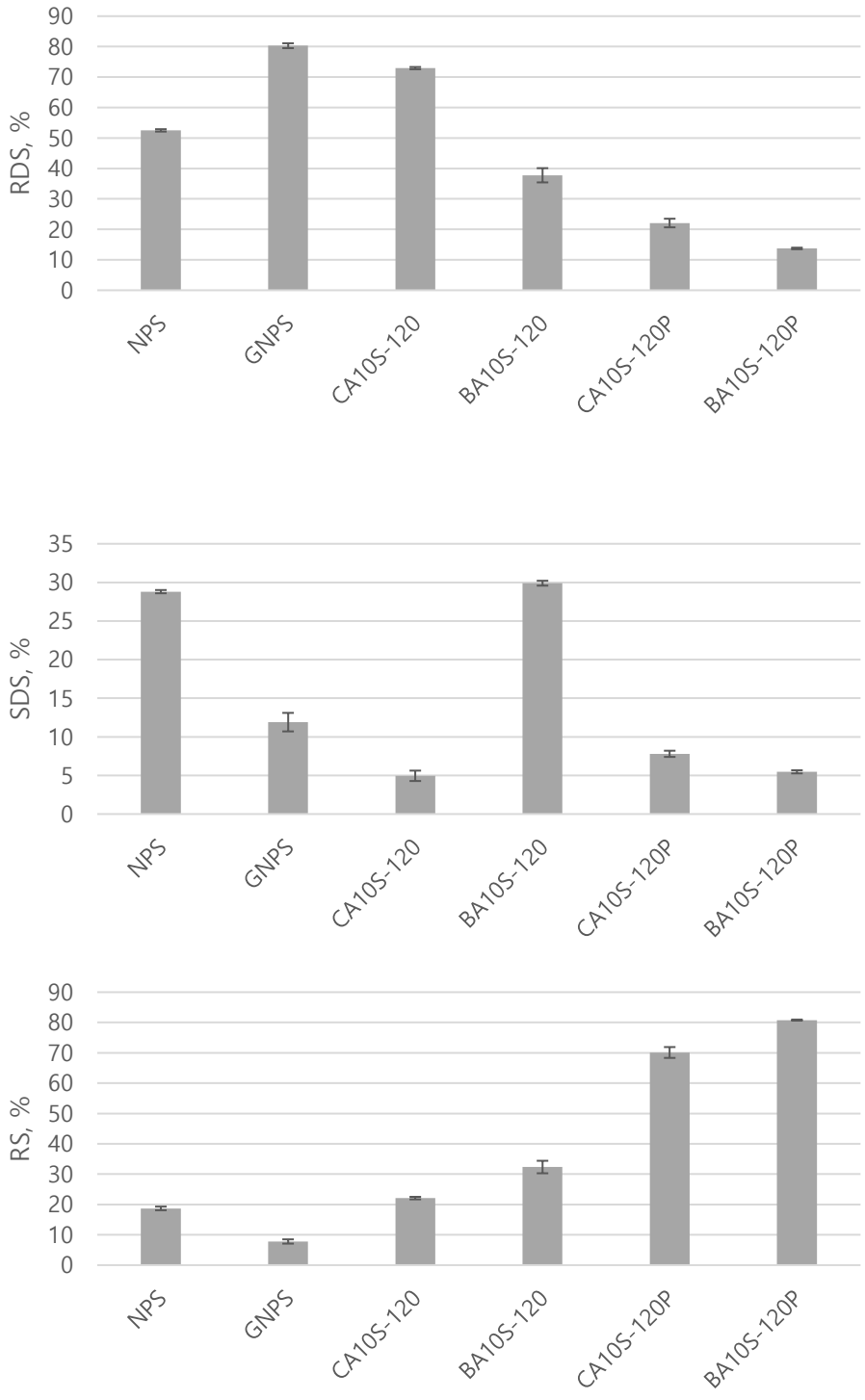

Figure 4.20 Effect of Cross-linking agents and Pullulanase on the Digestion Profiles of Pea Starch Samples

Table 4.3 and Figure 4.21 show the digestion profile of starch samples modified with epoxidized vegetable oils. Unlike the previous results using CA and BTCA, there was no change in RS content in any epoxidized pea starch sample. On the contrary, the fraction of SDS dramatically decreased while RDS content increased sharply. This may be due to the low epoxidation efficiency of the epoxidized oils. In order to overcome this problem, it is necessary to study more various methods for producing epoxidized oils and epoxidized pea starch samples.

Table 4.3 Digestion Profiles of Native and Epoxidized Pea Starch

Samples	Vegetable Oil	Reaction Time, **(hr)**	Digestion Profile (Englyst method)		
			RDS (%)	SDS (%)	RS (%)
EN0	N/A	N/A	52.49±0.39	28.84±0.21	18.67±0.60
EC12	Canola Oil	12	79.58±0.98	2.18±1.19	18.24±0.28
EC24	Canola Oil	24	79.04±1.19	2.45±0.86	18.51±0.39
ES12	Safflower Oil	12	79.06±1.22	2.09±0.98	18.85±0.42
ES24	Safflower Oil	24	80.87±1.30	2.76±1.00	16.37±2.11

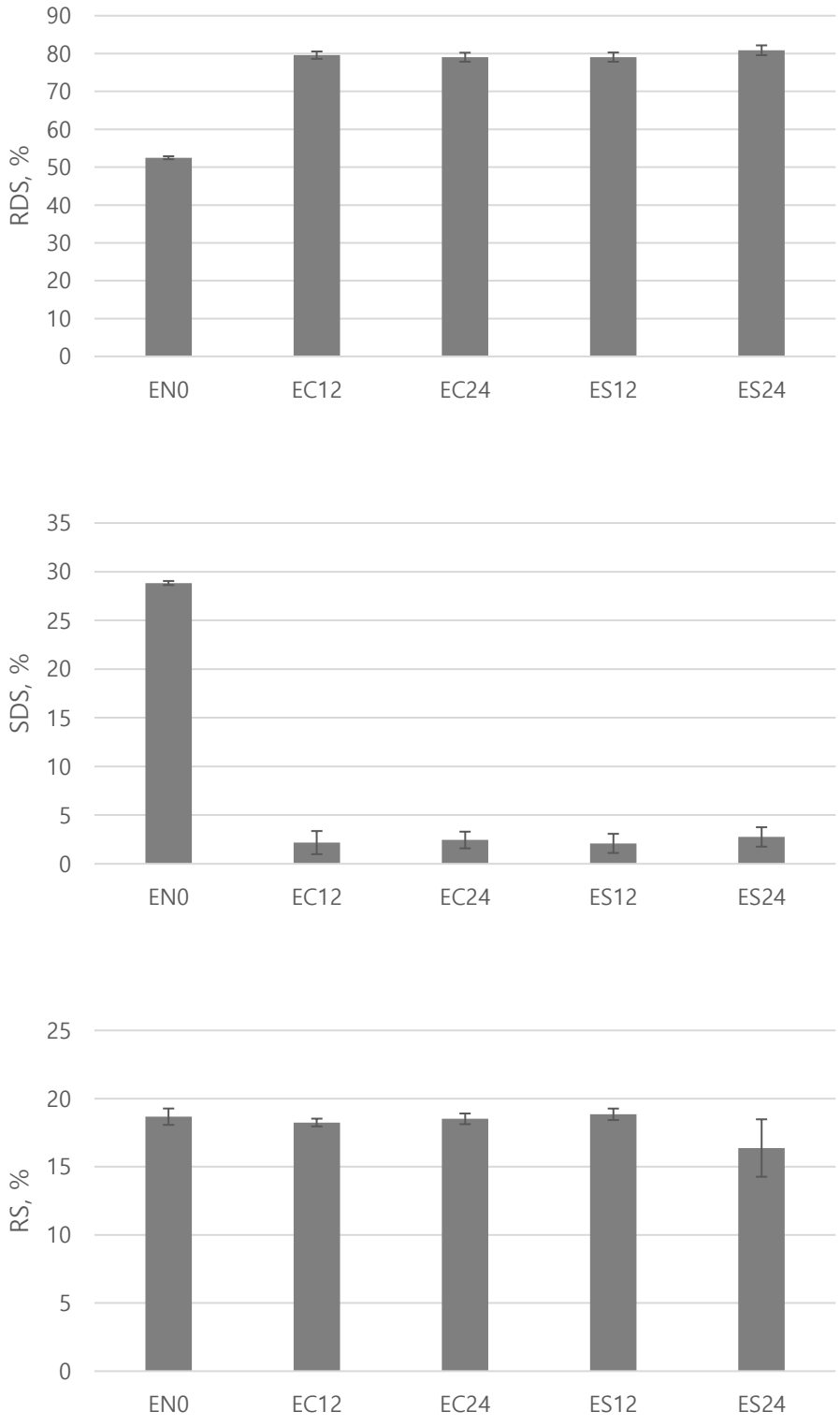

Figure 4.21 Effect of Epoxidized Oils on the Digestion Profiles of Pea Starch Samples

4.6 Total Starch Content

The measurement of total starch content was carried out to verify the high RS content of acid modified pea starch samples with/without pullulanase; total starch content is a measure of how much starch composition is present in the material using dimethyl sulfoxide (DMSO), heat, and enzymes. Figure 4.22 shows the overall results of total starch content for tested samples. CA10S-120 shows 73.3±0.51 % of total starch content, while BA10S-120P has 13.8±0.37 %. The overall trend for total starch content is inversely proportional to the fraction of RS. CA10S-120 showed the lowest RS content (22.11±0.41 %), but total starch content was the highest (73.3±0.51 %). Similarly, BA10S-120P showed the highest RS content (80.81±0.18 %), but the lowest total starch content (13.8±0.37 %). This is because acid modified pea starch samples had a high degree of cross-linking and so, they were resistant to digestion even in the total starch content method. Therefore, total starch content results for acid modified pea starch samples is considered evidence of high resistance to digestion as well as the Englyst results of digestibility.

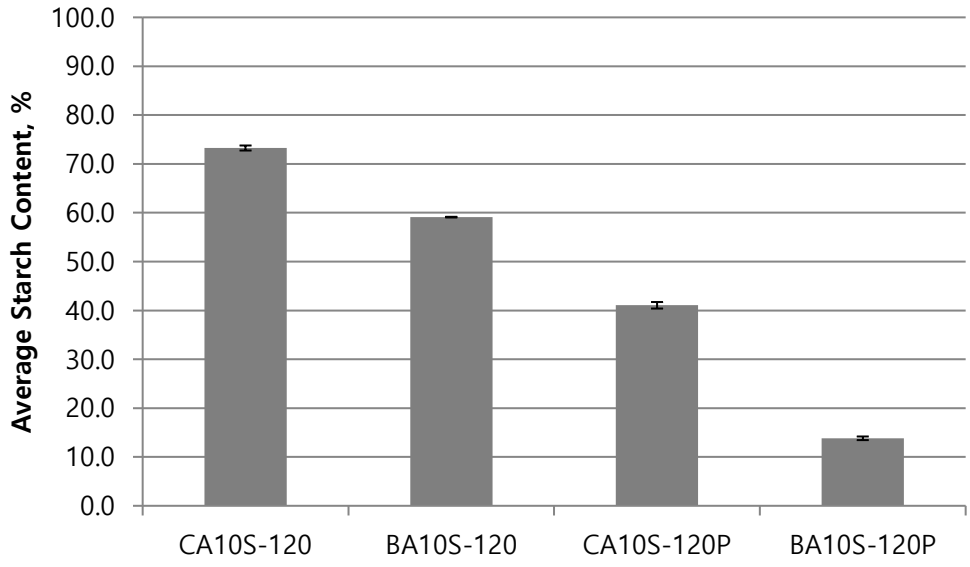

Figure 4.22 Total Starch Content of Various Acid Modified Starch Samples

4.7 Rapid Visco Analysis

The RVA pasting parameters of acid modified samples with/without pullulanase is shown in Figure 4.23. Unlike typical RVA curves (see Figure 4.24), viscosities for all samples were too low to compare. In the case of the enzyme treated samples, there may be a decrease in viscosity due to molecular weight reduction by chain scission. However, there was no change in viscosity for the acid modified samples. Only one assumption for this result is that the acid modified samples have a very high degree of cross-linking, so, they can not dissolve fully under the RVA operation temperature. This can

be seen in a similar context to the total starch content results, see Chapter 4.6.

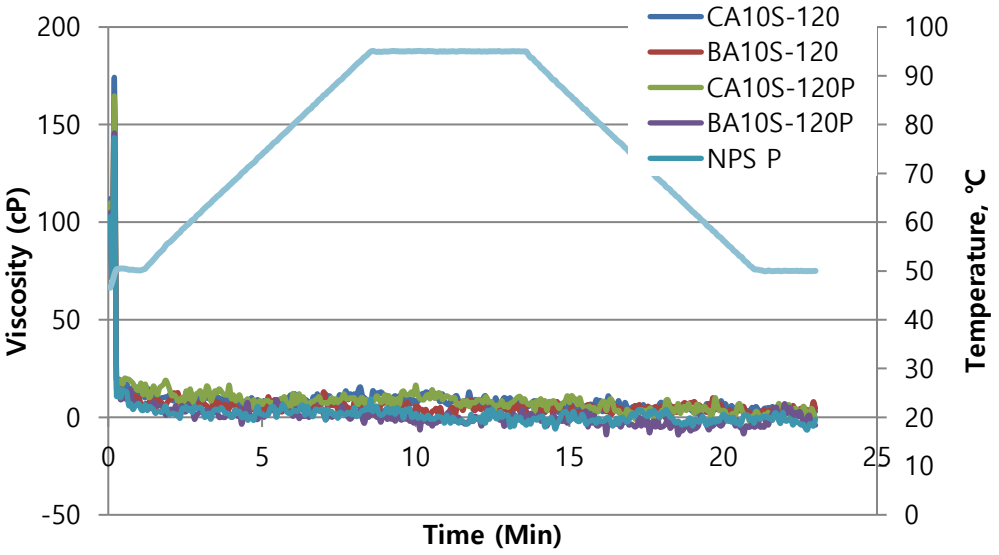

Figure 4.23 Rapid Visco Analysis Pasting Curve of Acid Modified Pea Starch Samples with/without Pullulanase

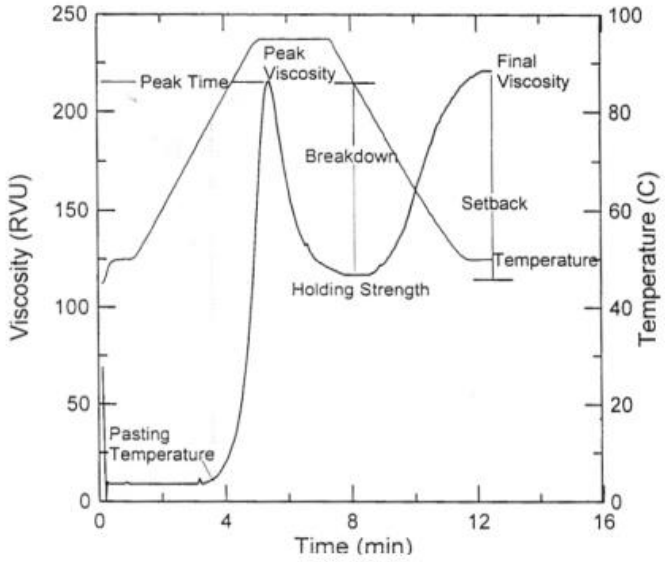

Figure 4.24 Typical Rapid Visco Analysis Pasting Curve (Perten Instruments Australia Pty. Ltd., 2010)

CHAPTER 5. CONCLUSIONS

5.1 Conclusions

Native pea starch samples and enzyme treated samples with/without heat moisture treatment, acid modification method with/without enzyme treated pea starch samples, physical modification method with acids treated pea starch samples, and epoxidized pea starch samples using vegetable oils were examined to seek better methods to increase the resistant starch content in this study. Heat moisture treated samples in both native starch and previously enzyme debranched starch samples proved to be too time consuming and offered little resistance to structural changes above the gelatinization temperature where cooking occurs. The intrinsic viscosity was dramatically decreased when pullulanase was used in comparison with the samples without pullulanase treated for all temperature conditions. However, the enzyme treated samples were even more susceptible to cooking temperature and ultimately, a chemical approach to improving digestion resistances was sought in this work with pea starch.

In acid modification methods, the optimized reaction temperature of citric acid was found at 120 ^{o}C. The starch samples showed some color change and declining FTIR absorbance at 1750cm^{-1} above this temperature. BTCA(1,2,3,4-Butanetetracarboxylic acid) showed slightly better crosslinked content than citric acid but perhaps not enough to

justify the less food-friendly molecule. BTCA treated samples demonstrated higher conversion and lower water solubility index than citric acid treated samples. In addition, sodium propionate as a catalyst showed a positive effect on WSI and conversion; samples with the catalyst showed slightly low WSI and high conversion than cross-linking agent only. BTCA cross-linking agent with sodium propionate reached the highest conversion (96.8±2.3 %) and the lowest WSI (13.1±2.0 %). RS content of these samples also increased far above native pea starch (CA10S-120: 22.11±0.41 %, BA10S-120: 32.35±2.06 %).

Debranched starch by the enzyme, pullulanase, has shown promise for improving the extent of crosslinking. Cross-linked with citric acid or BTCA, the intrinsic viscosity was increased while WSI decreased significantly. The cross-linking reaction for BTCA cross-linking agent with sodium propionate after enzyme treatment showed the lowest WSI (31.7±2.3 %) over citric acid. RS content of these samples was dramatically increased compared to using the cross-linking agent only, over 70% RS fraction (CA10S-120P: 70.12±1.79 %, BA10S-120P: 80.81±0.18 %).

Extruded samples with CA and BTCA have low WSI (TSE CA: 28.7±0.9 %, TSE BTCA: 22.4±1.1 %) than native pea starch sample (94.4±1.5 %). Although WSI of extruded samples were higher than

other modified samples, it was confirmed that acid-modified pea starch sample could be prepared by extrusion method.

Modified pea starch samples produced with epoxidized canola oil shows a new peak of epoxy group in FT-IR measurement. However, this peak did not appear in other samples. The epoxidized samples demonstrated higher WSI compare to acid modified samples. This is because of the low epoxidation efficiency of the self-produced epoxidized vegetable oils.

5.2 Recommendations

The effectiveness of using rapid bulk esterification by twin-screw extrusion process to improve enzyme resistance property of pea starches was relatively small compare to studies conducted by other modification methods. This is because only one extrusion condition was used in this study due to time constraints in the project. Thus, another configuration of screw design, different processing conditions such as temperature, feed rate, and total moisture content would be necessary to understand well the effect of processing conditions affecting the properties of pea starch samples. In addition, only water solubility index and conversion were measured to check the properties of extruded samples, in this study. However, more analysis studies such as digestibility and pasting property on extruded samples are necessary to know the exact properties of pea starch samples.

Another suggestion is that further studies on the effect of pullulanase should be conducted. Although some analysis were performed to investigate how the debranching enzyme act during the reaction, the analysis were not enough to prove the effectiveness of using debranching enzyme. Thus, additional characterization methods, such as XRD, NMR should be employ to understand the relationship between the structure and functional properties of debranched pea starches by pullulanase.

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APPENDICES

Appendix A. Gelatinization temperature of native pea starch

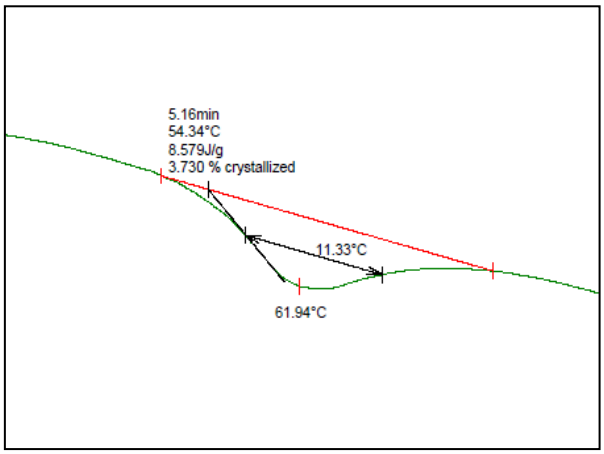

Figure A.1 Gelatinization temperature of native pea starch with 30% of moisture content

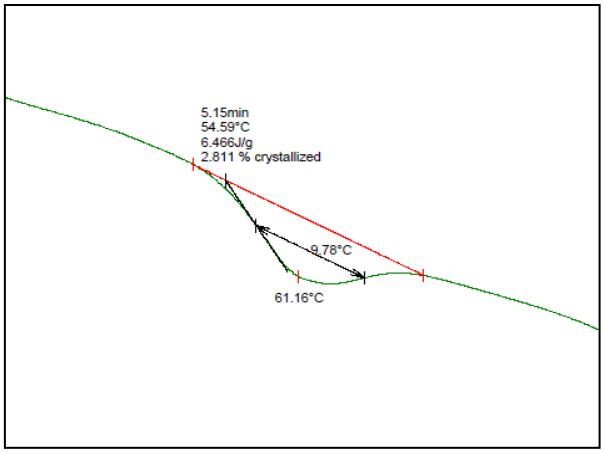

Figure A.2 Gelatinization temperature of native pea starch with 50% of moisture content

Appendix B. Thermal properties and FT-IR results of native pea starch and enzymatic treatment pea starch samples with/without HMT

Table B.1 Thermal properties and FT-IR($1045cm^{-1}/1022cm^{-1}$) of starch prepared with native pea starch and enzymatic treatment with/without HMT

Sample	Reaction Temperature (℃)	Reaction Time (hr)	HMT	Pullulanase (ml)	ΔH (J/g)	Melting Peak (℃)	FT-IR ($1045cm^{-1}/1022cm^{-1}$)
NPS-50-1	50	1	N/A	N/A	56.0	150.2	0.72
NPS-50-24	50	24	N/A	N/A	69.4	157.0	0.76
NPS-60-1	60	1	N/A	N/A	23.3	151.1	0.73
NPS-60-12	60	12	N/A	N/A	21.9	171.4	0.80
NPS-60-24	60	24	N/A	N/A	21.9	188.6	0.77
NPS-70-1	70	1	N/A	N/A	30.7	144.7	0.68
NPS-70-24	70	24	N/A	N/A	22.3	161.3	0.60
NPS-H-50-1	50	1	O	N/A	97.3	147.0	0.54
NPS-H-50-24	50	24	O	N/A	82.2	178.8	0.61
NPS-H-60-1	60	1	O	N/A	87.7	154.1	0.66
NPS-H-60-12	60	12	O	N/A	21.3	207.5	0.70
NPS-H-60-24	60	24	O	N/A	52.9	192.7	0.65
NPS-H-70-1	70	1	O	N/A	50.8	194.1	0.72
NPS-H-70-24	70	24	O	N/A	76.2	156.8	0.72
ETS-50-1	50	1	N/A	3.5	28.4	177.1	0.87
ETS-50-24	50	24	N/A	3.5	29.4	185.8	0.89
ETS-60-1	60	1	N/A	3.5	88.3	172.3	0.58
ETS-60-12	60	12	N/A	3.5	20.0	151.5	0.51
ETS-60-24	60	24	N/A	3.5	52.1	139.7	0.62
ETS-70-1	70	1	N/A	3.5	25.5	188.1	0.63
ETS-70-24	70	24	N/A	3.5	64.3	179.2	0.84
ETS-H-50-1	50	1	O	3.5	29.3	182.9	0.72
ETS-H-50-24	50	24	O	3.5	35.8	175.8	0.77
ETS-H-60-1	60	1	O	3.5	31.8	179.6	0.68
ETS-H-60-12	60	12	O	3.5	14.9	188.5	0.76
ETS-H-60-24	60	24	O	3.5	35.7	145.5	0.74
ETS-H-70-1	70	1	O	3.5	40.0	194.9	0.78
ETS-H-70-24	70	24	O	3.5	78.2	153.5	0.76