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In vitro fermentation of resistant canna starches †

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Abstract:
The indigestible residues remaining after amylase hydrolysis of native, acetylated (AC) and octenylsuccinylated (OS) canna starches were *in vitro* fermented with pig cecal contents. Fermentation of AC starch produced the highest amounts of total short-chain fatty acid (SCFA) (77.1 mM), followed by native (73.9 mM) and OS (64.2 mM) starches, respectively. Fermentation of AC and OS stacrhes generated acetic acid as a main SCFA, while butyric acid was a major component of SCFA derived from native starch. Fermentation of AC starch was slower than the other two starch samples. Slow fermtation rate of AC starch may be able to reach the more distal regions of colon, which might have a great impact on colonic health.

Keywords: Resistant canna starch; Short chain fatty acid; Acetylated starch; Octenyl succinylated starch

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1. Introduction

Resistant starch (RS) is the total amount of starch and the products of starch degradation that escapes digestion in the small intestine and reaches the large intestine [1]. RS is fermented by the colonic microflora producing short chain fatty acids (SCFAs) and gas (H_2, CO_2 and CH_4). The SCFA mainly acetic, propionic and butyric acids have been linked to a variety of physiological and health effect. *In vitro* studies as well as animal studies indicate that butyric acid has the potential to reduce risk factors that are involved in the development of colorectal cancer (inhibiting proliferation while increasing differentiation and apoptosis) [2]. The fermentation rate and relative molar ratio of SCFA are depending on amount and type of RS [3]. Chemically modified starches have been widely used in the food industry. Starches that have been chemically modified (cross-bonded with difunctional chemical reagents, ethers, esters, etc.) in such a way as to interfere with the action of digestive enzymes and therefore decrease their digestibility. Acetylated starch (AC) is commonly used as thickener, stabilizer, and binder in the food industry. Octenyl succinylated starch (OS-starch) is a modified starch esterified with octenyl succinic anhydride (OS) to establish the starch granule with amphiphilic properties. The OS-starch has been used as emulsifier, encapsulating agent and fat replacer [4].

Canna starch, a kind of starch extracted from rhizomes of the edible canna plant (*Canna edulis* Ker.), is mostly used for preparing transparent starch noodles, a traditional food of Southeast Asia. There have been reported that native canna starch had high resistance to hydrolysis by α-amylase [5]. Canna starch and its derivatives such as acetylated and octenyl succinylated canna starches have been reported to contain more than 95% RS content [6]. Moreover, Wandee, *et al.* [7] reported that the fermentation of native, heat-moisture treated (HMT) and cross-linked canna starches with pig cecal contents produced higher amounts of butyric acid (39–57 mol%) than that of retrograded debranched canna starches (12–14 mol%). However, there has been no information on the SCFA production from acetylated and octenyl succinylated canna starches. Therefore, this study aimed to investigate the fermentability of acetylated (AC) and octenyl succinylated (OS) canna starches. These starch including native canna starch were digested with enzyme and then fermented with pig cecal content. SCFAs production and type of SCFA were examined as shown in Figure 1.

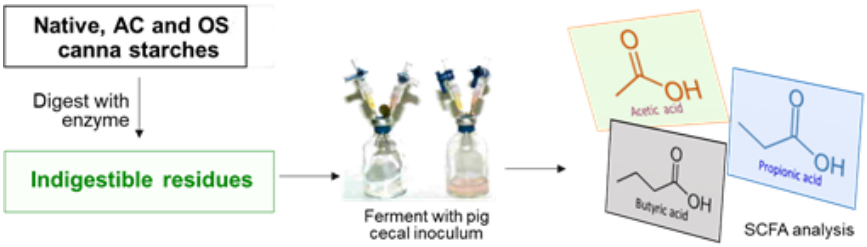

Figure 1. The procedure for *in vitro* fermentation of indigestible canna starches.

2. Materials and Methods

2.1. Raw materials

Eight-month-old rhizomes of edible canna plants were obtained from the Rayong Field Crops Research Center, Rayong, Thailand; the starch was isolated according to a procedure described by Puncha-arnon, *et al.* [8] Chemically modified canna starch samples were prepared according to the methods described in previous reports, as follows: acetylated canna starch (AC; DS = 0.06) [9] and octenyl succinylated canna starch (OS; DS = 0.017) [5].

2.2. Determination of resistant starch

The RS were determined according to the method of Wandee, *et al.* [7]. Starch (100 mg), 10 glass beads (0.5 mm diameter), 10 glass beads (0.5 mm diameter) and 10 mL of sodium acetate buffer (0.1 M, 4 mM $CaCl_2$, pH 5.2) were added to an Erlenmeyer flask, mixed and then added α-amylase (900 U; Sigma A-3173) and amyloglucosidase (7.5 U; Sigma P7545). The flask was incubated at 37 °C in a shaking water bath. After 120 min of incubation, 0.5 mL of samples were taken and the liberated glucose was determined by a glucose assay kit (GLUCOSE Liquicolor; HUMAN Diagnostics, Wiesbaden, Germany).

Indigestible residues remaining after 120 min of hydrolysis was recovered, washed twice with distilled water and freeze-dried. Duplicate dried samples were pooled, ground in a mortar, and passed through a 106 μm sieve for further fermetation investigation.

2.3. In vitro fermentation

In vitro batch fermentation was conducted according to the method of Wandee, *et al.* [7]. The inoculum was prepared from fresh cecal from three healthy pigs obtained from Fresh Meat Processing Co., Ltd. (Nakhon Pathom, Thailand). The cecal contents of individual donor were weighed and mixed with sterile fermentation medium (pH 7.2) in a ratio of 1:1 (w/w) and strained through four layers of cheesecloth. One hundred mg of indigested starch was added to 8 mL of fermentation medium in a 20 mL serum bottle. The bottle was sealed with a butyl rubber stopper and aluminum cap, and the headspace was flushed with N_2 for 3 min to maintain anaerobic conditions. The sample was then hydrated overnight at 4 °C. After equilibrating in a water bath at 37 °C for 1 h, 2 mL of inoculum was added to each bottle and the headspace was flushed again with N_2 for 1 min and then incubated in a shaking water bath (50 strokes/min) at 37 °C. Samples of the fermented broth (0.5 mL) were taken at 1, 2, 3, 5 and 7 d and immediately placed in a freezer at −20 °C to stop fermentation. A control containing no sample was used as a blank. The SCAF analysis was carried out using high-performance liquid chromatography (HPLC) [7].

3. Results and discussion

3.1. Resistant starch content

Resistant starch contents of native, AC and OS canna starches are presented in Table 1. RS content of native starch was 97.2%(w/w), while those of AC and OS canna starches were 99.5% and 99.5%, w/w, respectively. High resistance against enzyme hydrolysis of raw canna starches could be due to large size of canna starch granule (low surface area), smooth surface, and no pores on the surface [8]. The starch granules of AC and OS starches were still in intact granular form, typically round/oval-shaped granules with smooth surface, and were identical to the native starch [9].

Table 1. Amounts of resistant starch in canna starches

Samples	Resistant starch (%, dwb)
Native canna	97.2 ± 0.1
Acetylated	99.5 ± 0.0
Octenylsuccinylated	99.5 ± 0.5

Values are the mean of triplicate determinations.

3.2. Effect of inoculum donor on SCFA production

The intestinal microbiome of a healthy individual is a balanced community of different microorganisms, including bacteria, bacteriophages, viruses, archaea, and fungi [10]. Firstly, a pre-screening experiment was performed to gain information on the potential difference of three inoculum donors in response to native canna starch fermentation. Total SCFAs produced from indigestible residue of canna starch fermented with three inoculum donors after 7 d of fermentation are shown in Figure 2. Native starch fermented with inoculum from Donor #3 generated the highest total SCFA (98.7 mM), followed by Donor #1 and #2, respectively. Inoculum from Donor #1 and #3 produced acetic acid as the main product (65.0 and 78.5 mM, respectively), whereas Donor #2 generated butyric acid (40.7 mM) as the main product. Donor #2 showed an enhancement in

butyric acid production. Therefore, inoculum form Donor #2 has chosen for further investigation.

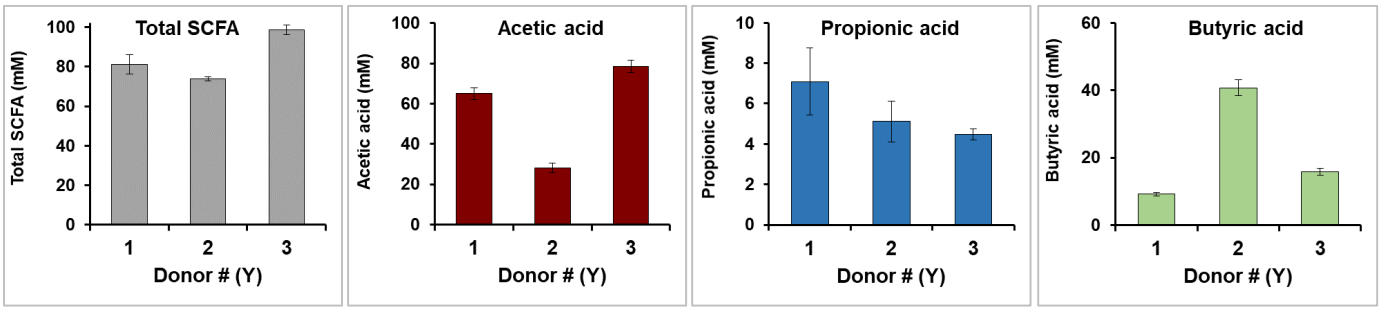

Figure 2. Amounts of total SCFA, acetic, propionic and butyric acids produced form native canna starch fermented with three different donors for 7 d.

3.3 Fermentability of indigestible canna starches

Total SCFAs, which is the sum of acetic, propionic and butyric acids, produced from 100 mg of indigestible residues of canna starches after 1, 2, 3, 5 and 7 d of fermentation are shown in Figure 3. For all substrates, the amounts of total SCFAs were very low for the first day. After 2 d, it was found that total SCFAs produced from native and OS starches increased to some extent, whereas AC starch needed more than 2 d for the bacteria in pig feces to start metabolizing it. This result was similar to a previous study which demonstrated that a certain time is needed for adaptation of these bacteria to the surrounding raw RS [7]. The large intestine is a complex ecosystem containing about 500 to 1000 different species [11]. Starch-degrading activity has been reported for three major phyla in the mammalian gut, Bacteroidetes, Firmicutes and Actinobacteria, including the genera Bifidobacterium, Butyrivibrio, Roseburia, Eubacterium and Bacteroides [12,13]. Each bacterial species in the gut has a typical pattern/mechanism of hydrolytic activities. Therefore, the type and amount of starch-degrading bacteria in the pig gut are considered to be important factors affecting raw RS hydrolysis.

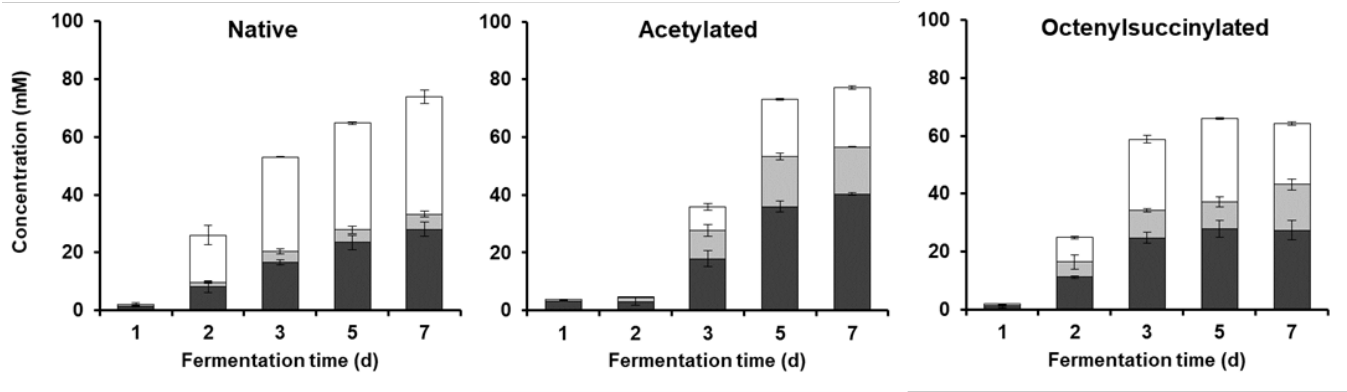

Figure 3 Amounts of acetic ( ), propionic ( ) and butyric ( ) acids produced by fermentation of indigested starch samples with pig cecal contents.

Total SCFA concentrations in fermentation media of all starches increased substantially after 3 d of fermentation. The results implied that bacteria containing amylolytic enzymes were activated, and their hydrolytic products can be used by other acid-

producing bacteria. Total SCFAs produced from AC starch was much lower than those from other starches. The substituted groups existing on the granular surface may impede the binding of amylase enzymes to the starch chains localized on the granule surface. As a result, AC starch has a slower fermentation rate than the OS starch. This result implied that amounts of substituted groups of AC starch was higher than of OS starch.

The total SCFAs produced from all samples increased consecutively until 5 d. After that, SCFAs generated from AC and OS starches reached a plateau, while native starch still continued generating the acids until the end of fermentation (7 d). Fermentation of AC starch produced the highest amount of total SCFAs (77.1 mM), followed by native canna (73.9 mM) and OS starch (64.2 mM), respectively. In term of fermentation products, AC starch is a promising prebiotic source since it can produce high SCFAs. *In vitro* experiments revealed that AC canna starch exhibited a slow fermentation rate; therefore, these starch are also expected to be slowly fermentable *in vivo* and will yield a certain amount of SCFAs along the distal colon.

Table 2. Amount of total SCFA and the ratio of acetic, propionic, and butyric acids produced by fermentation of indigested starch

Fermentation time (d)	Native		Acetylated		Octenyl succinylated	
	Total SCFA (mM)	Ratio of A : P : B	Total SCFA (mM)	Ratio of A : P : B	Total SCFA (mM)	Ratio of A : P : B
1	2.1 ± 0.8	74 : 00 : 26	3.8 ± 0.4	85 : 04 : 11	1.9 ± 1.1	65 : 09 : 26
2	26.0 ± 1.8	31 : 06 : 63	4.7 ± 1.4	66 : 28 : 06	24.9 ± 1.5	45 : 21 : 34
3	53.0 ± 1.5	31 : 07 : 62	35.9 ± 5.9	50 : 27 : 23	58.8 ± 3.8	42 : 16 : 42
5	64.8 ± 3.5	36 : 07 : 57	73.1 ± 3.2	49 : 24 : 27	66.1 ± 5.1	42 : 14 : 44
7	73.9 ± 1.1	38 : 07 : 55	77.1 ± 0.3	52: 21 : 27	64.2 ± 1.8	43 : 24 : 33

The SCFAs produced from the different indigestible residues after 1, 2, 3, 5 and 7 d showed differences in amount and proportion of each acid. For the first day, acetic acid was the main product of all substrates, and very small amounts of propionic and butyric acids were detected. Only native starch did not produce propionic acid. After 2 d, the amounts of all acids increased, and acetic acid was still the main product of all starches. For native starch, butyric acid was higher than propionic acid, i.e., the molar ratio of acetic (A): propionic (P): butyric (B) acids were 31:6:63 (Table 2). After 3 d, only AC starch produced acetic acid as the main product (ratio of A : P : B = 66:28:6). Amounts of acetic and propionic acids were comparable for OS starch (ratio of A : P : B = 42:16:42), whereas butyric acid produced from native canna starch remained unchanged as compared to 2 d of fermentation (62 mol%). Extending the fermentation to 5 d, the molar fraction of butyric acid produced from native canna starch slightly decreased (57 mol%). These results were in agreement with the previous reports in that RS fermentation generally results in a relatively higher butyric acid production in the order of 20-28 mol% compared to about 10-15 mol% for non-starch polysaccharides [2]. Fermentation of AC starch produced the highest fraction of acetic and propionic acids (24 mol%), followed by OS starch (14 mol%) and native canna starch (7 mol%). At the end of fermentation, AC starch produced the acetic acid as main product (52 mol%). One possibility, this acid might be released from acetyl groups existing on the AC starch granule by microbial action.

4. Conclusion

The results from this study revealed that raw AC and OS canna starches contain very high RS content (~99%), which can be fermented by pig cecal content to yield SCFAs. The amount of total SCFAs obtained from acetylated starch was higher, while butyric acid was lower than those of native and OS canna starches. Fermentation of AC starch was slower than the other two starch samples. It is known that the incidence of colon inflammation / cancer is high in the distal part of the colon. Therefore, the starch that has slow fermentation rate such as acetylated starch might be beneficial for gut health. To verify this supposition, fermentation of these starches in animal should be further studied.

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