J Sci Food Agric 1998, **76**, 221–225

Effect of Retrogradation, Pancreatin Digestion and Amylose/Amylopectin Ratio on the Fermentation of Starch by *Clostridium butyricum* (NCIMB 7423)

Carol-Ann Reid,[1,2]* Kevin Hillman[1] and Colin Henderson[2]

[1] Boyd-Orr Research Centre (BORC), Microbiology Division, Scottish Agricultural College, 581 King Street, Aberdeen AB24 5UD, UK
[2] The Robert Gordon University, School of Food and Consumer Studies, Kepplestone, Queens Road, Aberdeen AB9 2PG, UK

(Received 29 July 1996; revised version received 20 March 1997; accepted 19 June 1997)

Abstract: Using three different maize starches (maize, waxy maize and high amylose maize, containing 25%, 1% and 52% amylose, respectively) the influence of amylose/amylopectin content and of retrogradation on fermentation by the porcine caecal anaerobe *Clostridium butyricum* was assessed. Small intestine digestion was simulated using pancreatin before the starches were exposed to bacterial fermentation. It was found that retrogradation appeared to alter the extent of the fermentation and hence the amount of short-chain fatty acids produced, while pancreatin digestion appeared to alter the way in which the organism fermented the starch and hence the acetate/butyrate ratio. The amylose/amylopectin ratio seemed to have more influence on the way the starch was fermented by the bacteria after the starch had been subjected to digestion with pancreatic enzymes, but had less influence when the starch had been retrograded. © 1998 SCI.

J Sci Food Agric **76**, 221–225 (1998)

Key words: maize starches; *Clostridium butyricum*; fermentation; retrogradation; amylose/amylopectin

INTRODUCTION

The term 'resistant starch' refers to that portion of starch, and starch components, which are not digested in the small intestine and which passes into the large intestine to be fermented by the bacteria present (Cummings and Englyst 1987; Mathers 1992; Faisant *et al* 1993). The products of bacterial fermentation of resistant starch include short-chain fatty acids (SCFA), principally acetate, propionate and butyrate, and the gases hydrogen, carbon dioxide and methane (McNeil 1984; Abia *et al* 1993; Annison and Topping 1994).

It has been reported that untreated (native) starches from different botanical sources are fermented differently by the microflora of the large intestine, producing gas and SCFA in different proportions and at different rates depending on their origin (McBurney *et al* 1990).

In the present study three varieties of maize starches (waxy maize, maize and high amylose maize; 1%, 25% and 52% amylose, respectively) have been used to examine the influence that the amylose/amylopectin content might have on bacterial fermentation of starch. In addition, the influences of starch retrogradation (which is common in cooked and chilled foods) and

* To whom correspondence should be addressed at Microbiology Division, Scottish Agricultural College, 581 King Street, Aberdeen AB24 5UD, UK.

 J Sci Food Agric 0022-5142/98/$17.50. Printed in Great Britain

pancreatin digestion (which simulates small intestine digestion) on the fermentability of the starches has been examined. These *in vitro* studies were carried out using a known amylolytic species of bacteria, *Clostridium butyricum* AL (NCIMB 7423), which has been identified as a major degrader of starch residues (resistant starch) in the porcine large intestine (Baker *et al* 1950).

MATERIALS AND METHODS

Source of bacterial cultures and starches

Pure cultures of *Clostridium butyricum* (7423) were obtained from the National Collections of Industrial and Marine Bacteria Ltd (NCIMB, Aberdeen, UK) and were maintained throughout in Hungate tubes containing nutrient broth (Unipath, Basingstoke, UK), cooked meat granules (Lab M, Bury, UK) and 10 g litre^{-1} potato starch (Sigma, Poole, UK). Maize starch (BDH, Lutterworth, UK), waxy maize starch (National Starch and Chemical, Manchester, UK), and high amylose maize starch (BDH) were used throughout.

Preparation of native starches for bacterial fermentation

Starch suspensions (5 g litre^{-1}) were prepared in nutrient broth. These were heated to boiling point and cooled under anaerobic gas mixture (CO_2 : N_2 ; 50 : 50). This was comparable to the level of CO_2 found in the lower intestine of the pig (Jensen and Jørgensen 1994). The media were dispensed anaerobically in 5 ml amounts into Hungate tubes containing 1 g of cooked meat granules. The tubes were sealed with a butyl rubber septum and a screw cap lid and autoclaved at 121°C for 15 min to sterilise. The pancreatin digested form of the starches were prepared by adding 50 mg porcine pancreatin (Sigma) to 100 ml of a 100 g litre^{-1} starch suspension in distilled water. This was incubated at 40°C for 30 min and then centrifuged for 15 min at 6000 $\times g$. The supernatant liquid was discarded and the sedimented starch resuspended in distilled water and recentrifuged. The final pellet was resuspended in 100 ml distilled water to give a 100 g litre^{-1} starch suspension. This was added to prepared nutrient broth to give a final concentration of 5 g litre^{-1}. This was dispensed anaerobically and sterilised as above.

Preparation of retrograded starches for bacterial fermentation

To prepare the retrograded starches, 100 g litre^{-1} aqueous suspensions were autoclaved at 121°C for 15 min. These were cooled and stored at 4°C for 3 days to allow gel formation (with retrogradation) to occur. When required the gels were heated to 60°C to disperse and then aliquots added to nutrient broth to produce a 5 g litre^{-1} suspension. After heating to boiling point and cooling under anaerobic gas mixture, 5 ml were dispensed anaerobically into Hungate tubes containing 1 g of cooked meat granules. The tubes were sealed and sterilised at 121°C. Once cooled, the tubes were stored at 4°C for 3 days prior to use. Pancreatin digestion of the starches was carried out as above, adding 50 mg of porcine pancreatin to 100 ml of 100 g litre^{-1} retrograded starch gel. This gel had to be macerated prior to centrifugation. The residual retrograded starch was then diluted to 100 ml with distilled water and added to prepared nutrient broth to form a 5 g litre^{-1} starch suspension. This was then dispensed in the same way as the native pancreatin digested starches.

Bacterial cultures and fermentation measurements

The gas pressure in each Hungate tube was equilibrated to atmospheric pressure by adding the anaerobic gas mixture from a syringe. They were inoculated by adding 0·2 ml of a 24 h culture of *C butyricum*, which contained approximately 10^7 colony forming units (cfu). The experiment consisted of triplicate samples of each of the three starches subjected to each of the four treatments. All incubations were carried out for 48 h at 39°C. During the incubation, gas production was measured at 18, 24, 42 and 48 h intervals by allowing the gas in the tubes to expand into glass syringes. After 48 h, a final pH was recorded and a sample retained for SCFA analysis.

Analysis of SCFA by HPLC

To 1 ml samples, 0·2 ml of deproteinising reagent (comprising 200 g litre^{-1} metaphosphoric acid in 0·38 M sulphuric acid and containing 2·5 g litre^{-1} 2-ethyl-*n*-butyric acid as internal standard) was added. The samples were left to settle for 30 min at room temperature and then the liquids filtered through a 0·45 μm PTFE filter (Whatman, Maidstone, UK) into a Chromacol autosampler bottle. A Bio-Rad HPX-87H column and a Shodex SE-61 detector were used to perform HPLC analysis on the samples. The mobile phase was 5 mM sulphuric acid, flow rate 0·5 ml min^{-1} and column temperature 50°C. Of the principal SCFA known to be produced from fermentation of carbohydrates in the colon (Annison and Topping 1994), only acetate and butyrate were detected in samples from these incubations.

Statistical analysis

Statistical analysis (ANOVA) was performed on the triplicate data obtained using the MINITAB statistical package (Ryan *et al* 1985).

RESULTS

Influence of amylose/amylopectin ratio

Fermentation of the native starches varied with amylose/amylopectin content, although the differences were generally not significant. Production of SCFA (Table 1), gas (Fig 1) and final pH (Table 2), show a decreasing extent of fermentation of the three starches in the order maize > waxy maize > high amylose maize (HAM). Despite these variations the acetate/butyrate ratios were the same at 0·2.

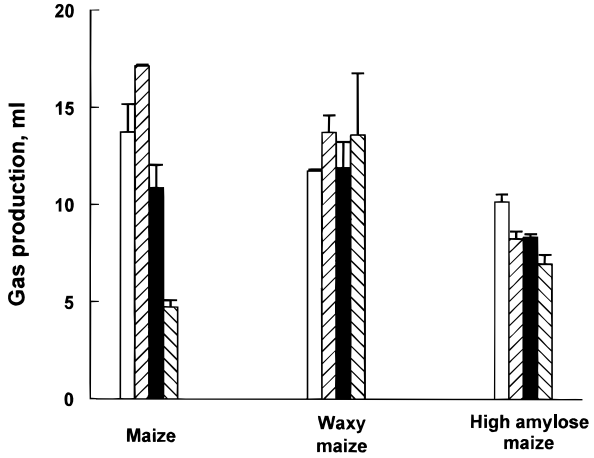

Fig 1. Total gas production (ml) by *C butyricum* when grown in media containing 5 g litre^{-1} of three maize starches after various treatments. Incubations were carried out for 48 h at 39°C. Bars represent mean + SEM of triplicate data. Lower error bars omitted for clarity. □, Native starch; ▨, native pancreatin digested starch; ■, retrograded starch; and ▧, retrograded pancreatin digested starch.

Pre-treatment of the native starches with porcine pancreatin (NPD) appeared to exaggerate the differences due to the amylose/amylopectin ratios but did not change the order of preference shown above. This was best indicated by the SCFA production (Table 1) and final pH (Table 2), where all three starches showed significant differences ($P < 0{\cdot}05$).

When the starches were subject to retrogradation before fermentation the *C butyricum* showed a preference for waxy maize over the two other starches, although the differences were not significant (Tables 1 and 2 and Fig 1). *Clostridium butyricum* showed no clear preference between the other two starches.

Fermentation of the retrograded starches after pancreatin digestion resulted in significantly different SCFA production from each starch, in the order waxy maize > HAM > maize (Table 1). In this instance the acetate and butyrate productions from waxy maize were much higher than from the other two starches. This was reflected in the corresponding final pH (Table 2) and gas data (Fig 1).

Influence of retrogradation and pancreatin digestion

Pancreatin digestion of the native starches (NPD) increased acetate production from maize and waxy maize and significantly decreased butyrate production from HAM. The increase in butyrate observed in the case of the pancreatin treated maize starch was not significant (Table 1). The acetate/butyrate ratios for all three NPD starches appeared similar at approximately 0·40, which was double that obtained for the starches in the native form. Despite the similarity in the acetate/butyrate ratios in the NPD starches, the gas production

TABLE 1
Production of SCFA (mM) from maize starches by *C butyricum* after 48 h incubation at 39°C (mean ± SEM, $n = 3$)[a,b]

		Starch treatment			
		Native	*NPD*	*Retrograded*	*RPD*
Acetate	Maize	$8{\cdot}17 \pm 1{\cdot}77^{a}$	$19{\cdot}26 \pm 0{\cdot}50^{b}_{x}$	$4{\cdot}89 \pm 0{\cdot}64^{a}$	$5{\cdot}94 \pm 0{\cdot}06^{a}_{x}$
	Waxy maize starch	$6{\cdot}34 \pm 0{\cdot}13^{a}$	$12{\cdot}99 \pm 1{\cdot}08^{b}_{y}$	$7{\cdot}00 \pm 1{\cdot}13^{a}$	$21{\cdot}50 \pm 0{\cdot}71^{c}_{y}$
	HAM	$5{\cdot}48 \pm 0{\cdot}41^{ab}$	$7{\cdot}65 \pm 0{\cdot}80^{bc}_{z}$	$4{\cdot}99 \pm 0{\cdot}18^{a}$	$8{\cdot}00 \pm 0{\cdot}29^{c}_{z}$
Butyrate	Maize	$37{\cdot}19 \pm 3{\cdot}73^{a}$	$47{\cdot}00 \pm 1{\cdot}06^{a}_{x}$	$23{\cdot}10 \pm 3{\cdot}24^{b}$	$13{\cdot}12 \pm 0{\cdot}21^{c}_{x}$
	Waxy maize starch	$33{\cdot}60 \pm 0{\cdot}96^{a}_{x}$	$33{\cdot}36 \pm 0{\cdot}39^{a}_{y}$	$29{\cdot}97 \pm 3{\cdot}89^{a}$	$53{\cdot}57 \pm 4{\cdot}89^{b}_{y}$
	HAM	$30{\cdot}01 \pm 1{\cdot}00^{a}_{y}$	$21{\cdot}69 \pm 1{\cdot}79^{b}_{z}$	$19{\cdot}76 \pm 0{\cdot}50^{b}$	$18{\cdot}84 \pm 1{\cdot}20^{b}_{z}$
Ac/Bu ratio	Maize	$0{\cdot}21 \pm 0{\cdot}02^{a}$	$0{\cdot}41 \pm 0{\cdot}00^{b}_{x}$	$0{\cdot}21 \pm 0{\cdot}01^{a}_{x}$	$0{\cdot}45 \pm 0{\cdot}00^{c}_{x}$
	Waxy maize starch	$0{\cdot}19 \pm 0{\cdot}00^{a}$	$0{\cdot}39 \pm 0{\cdot}03^{b}$	$0{\cdot}23 \pm 0{\cdot}02^{c}$	$0{\cdot}38 \pm 0{\cdot}03^{b}_{y}$
	HAM	$0{\cdot}18 \pm 0{\cdot}02^{a}$	$0{\cdot}35 \pm 0{\cdot}00^{b}_{y}$	$0{\cdot}25 \pm 0{\cdot}01^{c}_{y}$	$0{\cdot}43 \pm 0{\cdot}01^{d}$

[a] Values within a row bearing different superscript letters are significantly different ($P < 0{\cdot}05$); values in columns within each dataset bearing different subscript letters are significantly different ($P < 0{\cdot}05$).
[b] Abbreviations: HAM, high amylose maize starch; NPD, native pancreatin digested starch; RPD, retrograded pancreatin digested starch.

TABLE 2
Final pH of culture media containing treated or untreated starch after 48 h incubation with *C butyricum* at 39°C (mean ± SEM, $n = 3$)[a,b]

	Starch treatment			
	Native	*NPD*	*Retrograded*	*RPD*
Maize	$5{\cdot}10 \pm 0{\cdot}06^{a}_{x}$	$4{\cdot}98 \pm 0{\cdot}02^{a}_{x}$	$5{\cdot}65 \pm 0{\cdot}05^{b}$	$5{\cdot}85 \pm 0{\cdot}03^{c}_{x}$
Waxy maize starch	$5{\cdot}20 \pm 0{\cdot}00^{a}_{x}$	$5{\cdot}19 \pm 0{\cdot}02^{a}_{y}$	$5{\cdot}52 \pm 0{\cdot}08^{b}_{x}$	$5{\cdot}09 \pm 0{\cdot}15_{y}$
HAM	$5{\cdot}43 \pm 0{\cdot}03^{a}_{y}$	$5{\cdot}59 \pm 0{\cdot}08_{z}$	$5{\cdot}81 \pm 0{\cdot}03^{b}_{y}$	$5{\cdot}78 \pm 0{\cdot}04^{b}_{x}$

[a] Values within a row bearing different superscript letters are significantly different ($P < 0{\cdot}05$); values in columns bearing different subscript letters are significantly different ($P < 0{\cdot}05$).
[b] Abbreviations: HAM, high amylose maize starch; NPD, native pancreatin digested starch; RPD, retrograded pancreatin digested starch.

(Fig 1) and total SCFA production data indicated that the fermentation pattern of the three starches was altered in different ways. With maize and waxy maize starches, gas and acetate production increased whereas with HAM starch, gas and butyrate production decreased following pancreatin digestion.

Retrogradation of native maize and HAM significantly reduced butyrate production but no significant effect was noted in waxy maize starch (Table 1). This effect was reflected in the gas production data (Fig 1). The acetate/butyrate ratios (approximately 0·2) were similar to those obtained from the native starches. Significantly higher pH values were noted for all three retrograded starches.

When the starches underwent the combined treatments of retrogradation and subsequent pancreatin digestion (RPD), different effects were noted with each. The maize starch showed a gas production which was much lower than that obtained with any other treatment. This effect was reflected in the data obtained for butyrate production (Table 1) and final pH (Table 2). The same treatments applied to the waxy maize starch resulted in significantly higher acetate and butyrate production (Table 1), although there was little effect noted with gas production (Fig 1). With the RPD HAM starch, the acetate and butyrate productions were similar to those observed with the NPD form, and the gas production was at its lowest. The acetate/butyrate ratios were similar to those obtained for the NPD forms of the starch, ie double the values obtained for either the native or retrograded forms.

DISCUSSION

Three starches containing different proportions of the major components, amylose and amylopectin, were used to assess the effect that retrogradation and digestion with pancreatic enzymes had on the fermentation of these two components. Maize was used as a control as it contains 25% amylose (National Starch), which is representative of an average starch composition. Waxy maize contains negligible amylose (~1%) (National Starch) and HAM contains approximately 52% amylose (Lineback 1984).

In the native form the amylose/amylopectin ratio had little effect on the extent of fermentation as shown by SCFA and gas analysis. The acetate/butyrate ratios were calculated to examine changes in the pattern of fermentation by *C butyricum*. No significant differences were observed between the acetate/butyrate ratios for the three starches in their native form, hence the amylose/amylopectin content does not appear to significantly influence the way in which *C butyricum* ferments these starches.

In comparison with the native forms of these starches, retrogradation altered the extent of fermentation overall for the maize and HAM starches, but had little effect on the waxy maize starch. This was expected as retrogradation is known to affect the amylose more than the amylopectin part of the starch (Filer 1988). Even where the amounts of gas and SCFA produced had been altered, the acetate/butyrate ratios appeared to be similar to those obtained with the native forms of the starches. The HAM starch however showed a significant change in fermentation pattern when the starch was retrograded. This may be due to the high amount of amylose contained in this starch causing a higher degree of retrogradation.

When starch is heated in aqueous suspensions the amylose portion leaches out of the granules (Filer 1988). Subsequent cooling results in the formation of a retrograded gel which is based on amylose rather than amylopectin (Filer 1988). Biliaderis (1991) suggested that this gel coats the outside of the disrupted structure with the relatively intact amylopectin portion inside. If pancreatin disrupts this layer of amylose sufficiently to allow entry by the bacteria then the non-retrograded amylopectin can still be fermented. An amylose gel model described by Leloup *et al* (1992) showed that the gel contained partially crystalline regions separated by

amorphous regions, and that these amorphous regions were easily hydrolysed by acid and enzymic treatments. If these models are representative of starch gels, pancreatin could hydrolyse the amorphous regions within the gel, allowing bacterial access to the amylopectin beneath. These models could support the observed increase in fermentation from the starches, particularly waxy maize starch, when they had undergone retrogradation followed by pancreatin digestion. Using these models, it would appear that the more amylose present in the granule, the greater the amount of gel coating after retrogradation. Therefore, with high levels of amylose, pancreatin may not be able to break up the gel sufficiently in order to allow access to the amylopectin portion beneath.

Pancreatin digestion of the native starches resulted in a doubling of the acetate/butyrate ratio produced by *C butyricum*, generally due to an increase in the amounts of acetate formed. Butyrate and gas productions were increased in the maize starch, but significantly decreased in the HAM starch. Fermentation of the high amylopectin starch (waxy maize) resulted in an increased gas production but no change in butyrate production. These results suggest that digestion with pancreatin has removed some of the fermentable material from the high amylose starch but has apparently increased the availability of both maize and waxy maize to fermentation by *C butyricum*. This effect is unlikely to be due to any residual pancreatic enzymes being fermented by bacteria since different effects were observed with each starch. The acetate/butyrate ratios produced for the pancreatin digested starches, both native and retrograded, are similar to those produced by fermentation of pure amylopectin by this organism under the same conditions (Reid *et al* 1996), suggesting that after pancreatin digestion, the substrate for fermentation is principally amylopectin.

These findings show that the extent of fermentation of maize starches by the porcine caecal anaerobe *C butyricum* was more affected by retrogradation than by pancreatin digestion. Pancreatin digestion however, had more influence on the way the starch was fermented (acetate/butyrate ratio) and also accentuated the differences due to the amylose/amylopectin content of the starches. It appears that starches high in the amylopectin fraction, ie waxy maize, are not only more resistant to retrogradation, but are more accessible to bacterial fermentation after they have been treated with pancreatic enzymes.

Although the above experiments were carried out using a pure culture of *C butyricum*, the principles observed may apply to monogastric hindgut fermentation of starches in general, in that starches which contain higher than average amounts of amylopectin may produce an enhanced fermentation in the colon.

ACKNOWLEDGEMENTS

Funding for this work was provided by the Scottish Office, Agriculture, Environment and Fisheries Department. One of us (C-AR) was in receipt of a joint postgraduate award from the Boyd-Orr Research Centre (BORC) members, SAC and RGU.

REFERENCES

Abia R, Buchanan C J, Saura-Calixto F, Eastwood M A 1993 Structural changes during the retrogradation of legume starches modify the *in vitro* fermentation. *J Agric Food Chem* **41** 1856–1863.

Annison G, Topping D L 1994 Nutritional role of resistant starch: chemical structure vs physiological function. *Ann Rev Nutr* **14** 297–320.

Baker F, Nasr H, Morrice F, Bruce J 1950 Bacterial breakdown of structural starches and starch products in the digestive tract of ruminant and non-ruminant mammals. *J Pathol Bacteriol* **62** 617–638.

Biliaderis C G 1991 The structure and interactions of starch with food constituents. *Can J Physiol Pharmacol* **69** 60–78.

Cummings J H, Englyst H N 1987 Fermentation in the human large intestine and the available substrates. *Am J Clin Nutr* **45** 1243–1255.

Faisant N, Champ M, Colonna P, Buleon A, Molis C, Langkilde A-M, Schweizer T, Flourie B, Galmiche J-P 1993 Structural features of resistant starch at the end of the human small intestine. *Eur J Clin Nutr* **47** 285–296.

Filer L J 1988 Modified food starch—an update. *J Am Diet Assoc* **88** 342–344.

Jensen B B, Jørgensen H 1994 Effect of dietary fiber on microbial activity and microbial gas production in various regions of the gastrointestinal tract of pigs. *Appl Environ Microbiol* **60** 1897–1904.

Leloup V M, Colonna P, Ring S G, Roberts K, Wells B 1992 Microstructure of amylose gels. *Carbohydr Polym* **18** 189–197.

Lineback D R 1984 The starch granule—organisation and properties. *Baker's Dig* **13 Mar** 16–21.

Mathers J C 1992 Energy value of resistant starch. *Eur J Clin Nutr* **46** (Suppl 2) S129–S130.

McBurney M I, Cuff D J, Thompson L U 1990 Rates of fermentation and short chain fatty acid and gas production of six starches by human faecal microbiota. *J Sci Food Agric* **50** 79–88.

McNeil N I 1984 The contribution of the large intestine to energy supplies in man. *Am J Clin Nutr* **39** 338–342.

Reid C-A, Hillman K, Henderson C, Glass H 1996 Fermentation of native and processed starches by the porcine caecal anaerobe *Clostridium butyricum* (NCIMB 7423). *J Appl Bacteriol* **80** 191–198.

Ryan B F, Joiner B L, Ryan T A 1985 *Minitab Statistical Software* (2nd edn). Minitab, Boston, MA, USA.