Polysaccharide from Dry Navy Beans, *Phaseolus vulgaris*: Its Isolation and Stimulation of Clostridium perfringens

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A microbiological assay is described for determining gas produced by *Clostridium perfringens* (Veillon and Zuber) Holland from materials believed to be flatulent. A rationale for its use is given and analyzed. The fractionation and the results of the assay for several components of lima beans, *Phaseolus lunatus* L., and navy beans, *P. vulgaris* L., are given. In particular, a polysaccharide was isolated from dried *P. vulgaris* seeds. Only about one-seventh as much could be isolated from green *P. lunatus* seeds as from *P. vulgaris* seeds. The polysaccharide stimulates voluminous gas production by *C. perfringens* and meets all criteria of the rationale for flatulent subjects. It has not been tested on humans; however, data from the microbiological assay correlate well with human results for green and dry lima beans and navy beans.

There has been considerable interest in determining the nature of the flatulence factor of the common dry bean, *Phaseolus vulgaris* L.; lima bean, *P. lunatus* L.; and soy bean, *Glycine max* Merr. (3, 7–9, 12). Yet a positive identification of a factor has not been made for any of these species.

Progress has been slow because the ultimate assay must be performed in man, and human experiments are susceptible to uncontrollable factors such as emotions (3) and lung congestion (7). As a result, other organisms have been tried including dogs and bacteria (9, 12).

Richards et al. (9) first suggested a microbiological assay. Their methods suggested to us that flatulence ought to be caused by numerous foods known to be nonflatulent. The apparatus they used also proved to be very susceptible to mechanical error.

We developed a more precise apparatus for measuring gas from anaerobic cultures and formulated the medium described in this report. The assay described uses beans reported to be low in flatulence as a control. The rationale of our assay, including the assumptions that we have made, is discussed. Use of the assay resulted in the fractionation of a polysaccharide from California small white beans (*P. vulgaris*).

**MATERIALS AND METHODS**

Bioassay. The bioassay was accomplished by measuring the volume of gas produced by *Clostridium perfringens* (Veillon and Zuber) Holland (ATCC 3629). The basal medium contained 0.5 ml of liquid thioglycolate medium (4), 50 mg of finely ground California small white beans, and 10 ml of 0.1 M succinate (Na+, pH 6.0). The test media contained 30 mg of the bean fraction to be tested in addition to the basal medium. Autoclaving of the media at 15 psi for 20 min provided the only cooking for the bean powder and for fractions that had not previously been cooked. To assure a vigorous inoculum, 10 ml of thioglycolate medium was inoculated and incubated overnight at 35 C. In the morning, the entire tube was used to inoculate 30 ml of thioglycolate medium in a bottle. After the bottle had been allowed to incubate for 4 hr at 35 C in vacuo, it was used to inoculate the test medium.

The apparatus used for measuring gas consisted of a fermentation vessel and a gas buret attached to a manifold that was attached to a leveling bulb (Fig. 1). The fermentation vessel is a tube of about 13 ml capacity fitted with a $\frac{1}{2}$ 19/38 male joint. A glass bead was placed in the bottom to aid agitation. The gas buret consisted of an inverted 10-ml pipet fused to a $\frac{1}{2}$ 19/38 female joint with a side arm extending to just above the level of the medium. The lower end was sealed and openings were sawed in the side. The tip of the buret was capped with a 7-mm serum cap. The buret, manifold, and leveling bulb were filled with washed Hallikainen JY400 oil, a very light bath oil. The connections from the gas-buret side arm to the manifold and leveling bulb were made with polyvinyl chloride tubing. The manifold had a stopcock at the attachment for each of 10 gas burets and one further opening for the leveling bulb. A separatory funnel was used for the leveling bulb. The entire
apparatus was shaken in a modified Warburg bath set at 44°C.

Gas production was measured for 3 days. A blank containing the thioglycolate medium, bean powder, buffer, and inoculum (but no bean fraction) was always run. A hypodermic syringe could be used to obtain gas samples for analysis and to reset the buret to zero if more than 10 ml of gas was produced.

The assay was checked for linearity and reproducibility with several substrates. As determined by the standard deviation, it reproduced very reliably (Table 1). The assay was also linear within the limits of error expected (Table 2).

![Diagram of fermentation tube and gas buret.](http://aem.asm.org/)

**TABLE 1. Reproducibility of microbiological assay for several substrates**

| Substrate                      | Sample mean (ml of gas) | Standard deviation | Deviation per cent of mean |
|-------------------------------|-------------------------|--------------------|---------------------------|
| CSW polysaccharide A-1, 30 mg  | 13.00                   | 0.388              | 2.98                      |
| CSW polysaccharide A-1, 50 mg  | 15.00                   | 0.650              | 4.33                      |
| Whole beans, 50 mg             | 10.06                   | 0.514              | 5.12                      |

* California small white polysaccharide A-1 plus basal medium.

**TABLE 2. Linearity of the assay with two substrates at two concentrations each**

| Substrate                        | Milliliters of gas |
|----------------------------------|--------------------|
| California small white polysaccharide A-1 | 3.06 5.41 |
| California small white whole bean  | 5.80 10.06         |

* Gas produced in addition to gas produced by basal medium.

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**BEANS**

300g DRY, MICROGRIND, 0.05 in. SCREEN

GRIND IN WARING BLENDOR 3 TIMES WITH 1500ml WATER

CENTRIFUGE

RESIDUE

SUPERNATANT

AUTOCLAVE, CENTRIFUGE

SUPERNATANT

HEAT PRECIPITATED

CONDENSE TO 1200ml

ADD 150ml TANNIC ACID,
ADJUST TO pH 4.0, CENTRIFUGE

SUPERNATANT

ADD EQUAL VOLUME OF
ACETONE, CENTRIFUGE

PROTEIN TANNATE

SUSPENDED IN SATURATED
CAFFEINE SOLUTION, CENTRIFUGE

SUPERNATANT

CAFFEINE TANNATE

POLYSACCHARIDE SUPERNATANT

PPT., POLYSACCHARIDE A-1

ADD EQUAL VOLUME OF
ACETONE, CENTRIFUGE

PROTEIN A-1 PPT.

SUPERNATANT

**FIG. 1.** Fermentation tube and gas buret. Fermentation tube with male $\phi$ 19/38 joint (A). Gas buret with serum stopper at top (B), side tubulation extending to bottom of joint, and female 19/38 joint. The tubulation is attached by polyvinyl tubing to an oil displacement manifold (C) and a leveling bulb (D).

**FIG. 2.** Fractionation scheme used to obtain the water-soluble fractions.

All of our dry beans were either given to us by E. L. Murphy or tested by him to insure that they were flatulent. Our green Fordhook beans were commercially mature when harvested.

**Fractionation.** Figure 2 shows our main fractionation scheme. The scheme in Fig. 3 is better for separating the water-insoluble fractions: the starch and screenings. However, it is more laborious than the scheme (Fig. 2) for extracting the water-soluble fraction.

The finely ground bean powder was prepared for the scheme in Fig. 2 by passing it through a Mikro-Samplmill with a 0.022-inch (ca. 0.05 cm) screen. The action of this machine allows only the passage of particles that are many times smaller than the screen mesh. About 55% of the dry bean powder passed through a 200-mesh screen, although a small amount would not pass a 100-mesh screen. Thus, few cells could remain intact. A batch of 300 g of bean powder was mixed with 1,500 ml of water in a Waring Blender for 1 min. The slurry was centrifuged, and the precipitate was extracted twice in the same manner.
used were: saturated phenol; n-butanol–pyridine–water (6:4:3); and ethyl acetate–pyridine–water (2:1:2). Silver nitrate-sodium hydroxide dips and p-anisidine were used as indicators.

RESULTS

The yields and protein content of the various bean fractions are shown in Table 3. Starch represents over one-third of the total dry weight of the beans. An additional one-fifth of the dry weight of the beans was caught on the 100-mesh screen. The screenings were primarily cellulose, hemicellulose, and other insoluble materials. Heat precipitated approximately another 10% of the dry matter of the beans. This precipitate was primarily protein. The first acetone precipitate was the smallest fraction recovered from either bean by the scheme of Fig. 3.

Each of the fractions was studied in corresponding pairs from green Fordhook and California small white beans. A greater gas production from a California small white bean fraction was considered to suggest the presence of the flatulence factor. C. perfringens is known to grow on starch, but we wanted to determine whether the starches of the two beans might differ in their ability to support gas production. No difference in gas production was found between the two starches (Fig. 4).

The screenings showed little sign of a differential response (Fig. 4). The heat precipitates of the two beans showed a differential response, but it was the green Fordhook bean that supported the greater production of gas. The supernatant from the acetone precipitation was not assayed. It would contain oligosaccharides, peptides, and smaller compounds.

The first acetone precipitates also gave differential response. The California small white fraction gave the greater gas per 30 mg. A variety of means was used to further fractionate the first acetone fraction. Ultraviolet measurements

![Diagram](https://example.com/diagram.png)

**Fig. 3.** Fractionation scheme used for the preparation of screenings, starch, and first acetone precipitate.

The supernatants were combined, autoclaved, cooled, and centrifuged. The supernatant was then condensed to one-fourth its volume in a cyclone evaporator. A 10% tannic acid (J.T. Baker) solution was added to the condensed liquid at the rate of one-tenth of its volume. Next, acetic acid was added until the pH fell to 4.0. This procedure removed the protein remaining after heating (Table 3), and some polysaccharide (6).

An equal volume of acetone was added to the supernatant to precipitate the remaining polysaccharide. The precipitate was washed with acetone, air-dried, and labeled polysaccharide A-1. All of the other solids collected at other stages were also washed with acetone and air-dried. Later the protein tannate was suspended in saturated caffeine solution and extracted to yield a solution of free protein. The solution was precipitated with an equal volume of acetone, and the precipitate was washed with acetone. The precipitate was labeled protein A-1.

The substituent sugars of the polysaccharide were chromatogrammed after hydrolysis at 100°C in 1 N H₂SO₄ for 2.5 hr. Chromatograms were run on pre-coated Cellulose F thin-layer chromatography plates (E. Merck AG, Darmstadt, Germany). Solvents

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**Table 3. Yield of various bean fractions from green Fordhook and California small white beans**

| Bean fraction          | Yield* | Protein (6.25 × N Kjeldahl) |
|------------------------|--------|---------------------------|
|                        | GF     | DV | CSW | GF | DV | CSW |
| Screenings             | 16.4   | 20.9 | 20.1 | 11.00 | 5.16 | 15.19 |
| Starch                 | 41.4   | 34.4 | 34.2 | 1.25  | 0.30  | 0.50  |
| Heat precipitate       | 11.7   | 2.3  | 9.1  | 64.50 | 38.91 | 79.44 |
| Tannate                | 3.8    | 19.7 | 4.7  | 64.31 | 64.62 | 67.56 |
| Solubles               | 13.5   |     |      |       |       |       |
| Polysaccharide A-1     | 0.5    | 0.7  | 3.7  | 0.63  | 9.01  | 3.88  |
| First acetone precipitate | 7.4 | 3.1  |      | 61.50 | 9.01  | 15.06 |

* Fraction per 100 g (dry weight) of beans. Abbreviations: GF, green Fordhook; CSW, California small white; DV, dry Ventura.
and Kjeldahl determinations suggested that the green Fordhook fraction was mostly protein, whereas the California small white fraction contains a large percentage of polysaccharide (Table 3). The need for a simple means of separating proteins and polysaccharides suggested the scheme in Fig. 2. It proved to be very successful (Table 3; Fig. 4 and 5).

The extracted residue of the scheme in Fig. 2 would be essentially a composite of the starch and screenings of the scheme in Fig. 3. The significance of gas produced from it would be obscured by the large amount of starch present. The heat precipitate and acetone supernatant were similar to the corresponding fractions of the scheme in Fig. 3; therefore, they were not of interest.

The California small white and green Fordhook protein A-1 fractions contained the proteins of the corresponding first acetone precipitates. Yields were not quantitative for the protein A-1 fractions; however, the tannate yields are indicative of the protein A-1 yields.

The polysaccharide A-1 fraction of California small white beans not only supported much more gas production per 30 mg than the corresponding green Fordhook fraction (Fig. 4), but it also was a much larger fraction of the bean (Table 3 and Fig. 5). These data correlate well with human flatulence measurements with the same beans (7, 10).

After we had shown a correlation between human flatulence and gas production from the polysaccharides A-1, from green Fordhook, and California small white beans (Fig. 5), data from a third bean were desired. Dry Ventura beans are intermediate in flatulence (7, 10). Figure 4 shows that polysaccharide A-1 from dry Ventura beans produced more gas in our assay. The yield of polysaccharide A-1 from dry Ventura was small (Table 3), so that the gas produced from the polysaccharide A-1 in 1 g of dry Ventura bean was intermediate between that of green Fordhook and California small white beans (Fig. 5). Thus the results from all three beans in our assay correlate with measurements of human flatulence.

The hydrolyzed polysaccharide A-1 of California small white beans contained the following sugars: arabinose, galactose, xylose, and glucose. No other monosaccharides were found.

**DISCUSSION**

The use of a microbiological assay requires some assumptions. The literature contained no list of assumptions for a microbiological flatulence assay, so it was necessary to develop a list to rationalize our results.

First we assumed that both a reference and a flatulent bean were necessary to the development of an assay. We desired beans that were as different as possible in flatulence but as taxonomically similar as possible. Green Fordhook (*P. lunatus*) was chosen as the nonflatulent control on the basis of work done on humans by Sanchez et al. (10). The California small whites (*P. vulgaris*) are a typical dry navy bean and were chosen as the flatulent bean because of available comparative data (7).

Hydrogen is the most characteristic gas of flatulence (3, 9), so the assay organism should pro-
roduce hydrogen. Richards et al. (9) working with dogs and Murphy (7) working with humans found that Mexiform and its constituent iodochlorohydroxyquinoline (Vioform) inhibited intestinal gas formation, particularly hydrogen formation, but 2,4-dinitrophenol did not. Mexiform stimulates intestinal aerobic and coliform bacteria in rats according to Eisman et al. (5). These facts suggested that coliforms and intestinal aerobes should not be used. The only major hydrogen-producing inhabitants of the gut that could not be eliminated (5, 7, 9) were the clostridia and the Bacteroides. Only clostridia were used by Richards (1965), since he would have killed all but the sporeforming bacteria by pasteurization, and his incubation procedures were anaerobic. We found significant gas production when pasteurized human feces were used to inoculate cooked ground beans, but not when unpasteurized feces were used. We also did an experiment in which we added Vioform to our bioassay. The control produced gas, although those containing Vioform produced none. Thus we established that our assay organism was inhibited by Vioform just as flatus was inhibited. The C. perfringens culture used, therefore, met all of the criteria for an organism responsible for flatus.

It seemed most likely that the basic assay as rationalized thus far would give many misleading results. As was expected when the beans were only ground, cooked, and assayed, green Fordhook beans supported more gas production than California small white beans (Fig. 4). Apparently the larger quantity of small molecules in the green Fordhook beans was responsible.

An additional assumption was necessary to obviate the interference by molecules that would easily be absorbed by the gut. We made the assumption that flatulence was not due to any small molecule. Contrary evidence from bacterial experiments has been published (8, 11); however, there is also evidence from human experiments that at most oligosaccharides account for only a small fraction of the problem (3). No evidence has been published that any other small molecules produce flatus.

A final assumption is that the flatulence factor is one or more chemical entities present in beans. In order for the assay to work, flatus may not be caused by a precursor that must be changed by the gut before C. perfringens can utilize it. In using a nonflatulent bean as a reference, we were making an implicit assumption that none of the nonflatulent beans not digested and absorbed by the gut is metabolized by C. perfringens. Conversely, a certain portion of the flatulent bean must not be absorbed by the gut, but must be metabolized by C. perfringens. For the success of the assay, it is necessary that the flatulent beans contain nothing that is both absorbed by the gut and metabolized by C. perfringens which is not also present in the nonflatulent bean. Even then the foregoing assumptions must all be true, i.e., that C. perfringens is the organism or is sufficiently like the organism that produces gas in vivo, that what we are looking for has not been thrown away in our fractionating procedure, and that the flatulent factor is a chemical or chemicals present in the bean. However, the nature of our polysaccharides makes it seem reasonable that it is a flatulence factor.

Another legume gum, guar gum, does not cause a body weight increase in proportion to the difference between the intake and fecal weights of rats (2). However, rats fed a neomycin supplement gained more weight and had lower fecal weights than those on a basal diet (1). It seems likely that most of the guar gum was metabolized by bacteria with the production of flatus. This is the effect that we would suggest for the bean polysaccharide.

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