Comparison of Hydrolysis Methods Used in Feed, Digesta, and Fecal Starch Analysis

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Determinations were made of α-linked D-glucose polymers (starch) in biological materials using an amyloglucosidase or 0.76 N HCl for hydrolysis, followed by oxidase analysis of glucose. Purified starches and substrates high in starch content gave similar results with either hydrolysis method. Feces and feeds containing 17% or more cellulose resulted in higher starch estimates with acid hydrolysis due to limited cellulose degradation by the dilute acid. Starch digestibility would tend to be underestimated if acid hydrolysis were used in feed and fecal starch analysis; however, errors were small (<1%) with a high starch diet.

Methods used for analyzing starch in animal digestion studies are varied and may show considerable variation among different laboratories employing the same techniques (Topps and Kay, 1969). Some of the more common methods in use were reviewed by MacRae and Armstrong (1968). Hydrolysis of starch in cereal grains to D-glucose by the method of AOAC (1960), followed by analysis for reducing sugars (Osman et al., 1970) or glucose (Frederick et al., 1973), has previously been used at this station. Since hydrolysis in weak HCl as called for by the AOAC procedure is nonspecific, some hydrolysis of nonstarch glucose polymers (such as cellulose) could conceivably occur, resulting in overestimation of starch in some substrates.

MacRae and Armstrong (1968) describe an enzymatic hydrolysis procedure using an amyloglucosidase produced by *Aspergillus niger* carrying the trade name "Agidex". Thivend et al. (1972) also describe an amyloglucosidase procedure for determination of starch, which recommends a shorter hydrolysis time than that of MacRae and Armstrong (1968) but requires pressure heating at 135 °C rather than refluxing at 100 °C to effect starch gelatinization. Amyloglucosidase is highly specific for α-linked D-glucose polymers and will hydrolyze these polymers completely to D-glucose (Pazur and Ando, 1959). This procedure virtually eliminates overestimation of starch in biological materials (feeds, feces, etc.) due to cellulose breakdown but is more time consuming than the AOAC (1960) procedure.

This investigation was carried out to compare enzyme (amyloglucosidase) hydrolysis and AOAC acid hydrolysis for analyzing the starch content of feed, digesta, and fecal material. A modification of the enzymatic procedure of MacRae and Armstrong (1968) is also described.

EXPERIMENTAL PROCEDURE

An apparatus was designed to meet the refluxing requirements for both acid and enzyme hydrolysis procedures. A six-place heating element (Labconco micro-Kjeldahl digester) was used to provide heat. Samples were weighed into beakers of the type used on the Goldfisch fat extractor, and 100-mL round-bottom boiling flasks were set up in series to serve as condensing units (Figure 1). Water circulation through each flask was supplied by two glass tubes mounted in a rubber stopper. The inlet tube was longer to carry the water to the bottom of the flask and the outlet tube exited near the top. It was necessary to restrain the rubber stoppers to prevent their expulsion due to the pressure created within the flasks during the refluxing. A rack was constructed to permit the simultaneous raising or lowering of six condensers. When the condenser flasks were lowered onto the beakers, each flask rested independently of the others.

The AOAC (1960) acid hydrolysis procedure was modified somewhat for these studies. A 0.5-g sample was refluxed at 100 °C for 2.5 h in 90 mL of 0.76 N HCl. The solution was then cooled to room temperature and 90% of the acid neutralized with 5.5 mL of 45% NaOH. The solution was filtered through E and D 515 filter paper and brought to volume with distilled water in a 250-mL volumetric flask.

The enzyme hydrolysis procedure of MacRae and Armstrong (1968) requires that the volume of the hydrolyzing solution be determined by weight. This method will henceforth be referred to as the gravimetric procedure. An alternative technique was devised that reduced the number of analytical weighings required and allowed the volume of the hydrolyzing solution to be determined directly. This method will be referred to as the volumetric procedure and is described below.

A 0.5-g sample was refluxed at 100 °C for 4 h in 50 mL of distilled water, followed by the addition of 50 mL of 0.2 M acetate buffer. Approximately 0.5 g of Agidex enzyme was added and the beaker covered with a watch glass. The sample was maintained at 60 °C for 24 h in a dry-heat gravity convection incubator. Following incubation, the sample was cooled to room temperature, and the solution filtered through E and D 515 filter paper into a 250-mL volumetric flask and brought to volume with distilled water.

Glucose oxidase was used to quantify D-glucose produced by either enzyme or acid hydrolysis. Calculations for D-glucose or starch concentration (dry basis) were identical for volumetric enzyme hydrolysis and acid hydrolysis:

$$\text{mg of D-glucose/g of sample} = (A_u/A_s)[(10C_sV)/(WR)] \quad (1)$$

A_u is the absorbancy of the unknown, A_s is the absorbancy of the standard, C_s is the concentration of the standard (mg/100 mL), V is the volume in milliliters, W is the sample weight in grams, and R is the D-glucose recovery factor expressed as a decimal. An accurate estimate of starch concentration is calculated by replacing the number 10 in the above equation by the number 9 and substituting a starch recovery factor for R. (Since the weight of the D-glucose incorporated into a starch molecule is increased by ~10% upon hydrolysis, due to the addition of water, it is necessary to multiply the D-glucose recovered by starch hydrolysis by 0.90 to convert D-glucose to starch equivalent weight.) For samples of equal weight that are brought to

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Table I. Mean D-Glucose Recoveries from Four Substrates Using either Volumetric or Gravimetric Enzyme Hydrolysis[a]

substrate	recovery, mg of glucose/g ± SD	
	volumetric hydrolysis	gravimetric hydrolysis
potato starch	1100 ± 6	1101 ± 5
sorghum grain	773 ± 4	774 ± 5
alfalfa hay	21 ± 2	23 ± 2
feces[b]	15 ± 2	17 ± 2

[a] Means of two runs of three replications each. [b] Feces from a steer fed a diet containing 63% sorghum grain, 15% cottonseed hulls, and 5% alfalfa hay.

the same volume, the second factor on the right side of eq 1 can be considered to be a constant, K, and the equation becomes

$$\text{mg of D-glucose (or starch)/g of sample} = (A_u/A_s)K \tag{2}$$

Feed, digesta, and fecal samples subjected to analysis were dried to constant weight and ground to pass through a 1-mm screen in a Wiley mill. Data were subjected to statistical analysis according to Steel and Torrie (1960).

RESULTS AND DISCUSSION

Volumetric vs. Gravimetric Enzyme Hydrolysis. The gravimetric enzyme hydrolysis procedure described by MacRae and Armstrong (1968) was modified to simplify the analytical process. The advantages of the volumetric method are (a) fewer weighings required per sample, (b) elimination of the use of paraffin, and (c) simplified calculations.

In the volumetric procedure only the sample must be weighed, whereas the gravimetric method requires three to four analytical weighings. Preliminary investigations established that considerable variation in the amount of Agidex (0.1–0.7 g) did not alter D-glucose recovery from starch hydrolysis of 0.5-g grain samples. Thivend et al. (1972) also reported that corn starch recovery was not altered with wide ranges in amyloglucosidase concentrations. A level $^1/_4$-tsp measure was found to supply ~0.5 g of Agidex and provided a convenient and rapid method for adding Agidex to large numbers of samples.

Paraffin was added in the gravimetric procedure to inhibit microbial growth. The volumetric method requires only that the beaker be covered with a watch glass to reduce evaporation. Microbial growth did not appear to be a problem in the volumetric procedure, as D-glucose recovery was 97.5% for both techniques.

The results of comparisons between gravimetric and enzyme hydrolysis of various substrates are presented in Table I. No differences were found in D-glucose recovery or magnitude of variation for any of the substrates tested. The D-glucose recovered from potato starch (adjusted for 97.5% D-glucose recovery) was essentially the same as the theoretical 1111 mg of D-glucose produced by hydrolysis of 1000 mg of starch.

Compared to the previously established gravimetric procedure, the volumetric hydrolysis method produced results of similar accuracy and was considered by this laboratory to simplify the analysis. Therefore, all subsequent analyses reported in this paper involving enzyme hydrolysis were performed using the volumetric method.

Enzyme Hydrolysis vs. Acid Hydrolysis. The acid hydrolysis procedure used at this station is a relatively rapid, simple method requiring only 2.5 h for refluxing, followed by immediate analysis for D-glucose. In contrast, enzyme hydrolysis requires a 4-h reflux period and a 24-h incubation. In these studies, D-glucose recovery from acid hydrolysis was similar to that of enzyme hydrolysis (96.3 vs 97.5%).

Table II. Mean D-Glucose Recoveries from Different Substrates Using Acid or Enzyme Hydrolysis[a]

substrate	recovery, mg of D-glucose/g ± SD	
	acid hydrolysis	enzyme hydrolysis
red milo starch	1099 ± 4	1106 ± 10
corn starch	1100 ± 10	1102 ± 3
potato starch	1096 ± 3	1100 ± 6
sorghum grain	772 ± 4	773 ± 4
alfalfa hay	33[b] ± 4	21[c] ± 2
feces[d]	31[b] ± 1	15[c] ± 2
cotton	11[b] ± 2	0[c] ± 0

[a] Means of two runs of three replications each. [b,c] Means on same line with unlike superscripts differ ($P <$ 01). [d] Feces from a steer fed a diet containing 63% sorghum grain, 15% cottonseed hulls, and 5% alfalfa hay.

Table III. Mean D-Glucose Recoveries from Varying Ratios of Grain and Hay Using Acid or Enzyme Hydrolysis[a]

% hay and grain in mixture		recovery, mg of D-glucose/g ± SD	
hay	grain	acid hydrolysis	enzyme hydrolysis
100	0	33[b] ± 4	21[c] ± 2
80	20	180[b] ± 2 (180)[f]	170[c] ± 3 (171)
67	33	277[d] ± 6 (277)	269[e] ± 3 (271)
50	50	400 ± 5 (402)	402 ± 6 (397)
33	67	526 ± 5 (528)	524 ± 7 (523)
20	80	622 ± 4 (624)	626 ± 11 (623)
0	100	772 ± 4	773 ± 4

[a] Means of two runs of three replications each. [b,c] Means on same line with unlike superscripts differ ($P <$ 0.01). [d,e] Means on same line with unlike superscripts differ ($P < 0.05$). [f] Figures in parentheses are calculated values based on 100% hay and 100% grain values as modified by varying hay/grain ratios.

MacRae and Armstrong (1968) reported substantial quantities of reducing sugar produced by hydrolysis in 0.36 N H_2SO_4. Since glucose oxidase was used following hydrolysis, nonglucose sugars were not considered a factor here. α-linked D-glucose polymers (i.e., starch) were considered to be the only source of D-glucose when hydrolysis was by enzymatic degradation, whereas other nonstarch compounds (i.e., cellulose) were considered potential D-glucose sources with acid hydrolysis.

Hydrolysis by either acid or enzyme gave similar results for D-glucose recovery and for magnitude of variation when the substrates were purified starches or sorghum grain (Table II). Cotton, composed of nearly pure cellulose, yielded 11 mg of D-glucose/g of sample with acid hydrolysis but none with enzyme hydrolysis. Cotton fibers subjected to acid hydrolysis were observed to have been reduced in length from 2–3 cm prior to hydrolysis down to 3–5 mm following hydrolysis. In contrast, enzyme hydrolysis produced no visual evidence of fiber disjunction.

Higher ($P < 0.01$) D-glucose recoveries were also obtained by acid hydrolysis when the substrates were alfalfa hay or fecal material. The hay contained ~26% cellulose and the fecal material, 17% cellulose. Acid hydrolysis of the hay and feces produced 12 and 16 mg of additional D-glucose, respectively, compared to enzyme hydrolysis.

Grain and hay were then mixed in varying ratios to test the consistency of starch overestimation with substrates varying in starch and cellulose contents (Table III). With up to 50% hay in the mixture (~13% cellulose), there were no differences in D-glucose recovery between acid

Table IV. Mean Starch Recoveries from Feed, Digesta, and Feces Using either Acid or Enzyme Hydrolysis

substrate	recovery, mg of starch/g ± SD			
	steer 1[a]		steer 2[a]	
	acid hydrolysis	enzyme hydrolysis	acid hydrolysis	enzyme hydrolysis
feed[b]	678 ± 12	681 ± 11	674 ± 2	670 ± 11
abomasal digesta[c]	188 ± 3	185 ± 3	357 ± 7	358 ± 3
small intestine digesta[c]	85[d] ± 2	72[e] ± 9	116[d] ± 3	107[e] ± 3
large intestine digesta[b]	79[d] ± 4	58[e] ± 3	212[d] ± 1	192[e] ± 1
feces[b]	46[d] ± 4	12[e] ± 1	201[d] ± 5	180[e] ± 1

[a] Steer 1 received a diet containing 5% alfalfa hay, 5% cottonseed hulls, and 80% steam-flaked sorghum grain; steer 2 received an identical diet but with dry-rolled sorghum grain. [b] Means of one run with three replications each. [c] Means of two runs with three replications each. [d,e] Means on same line within a steer with unlike superscripts differ ($P < 0.01$).

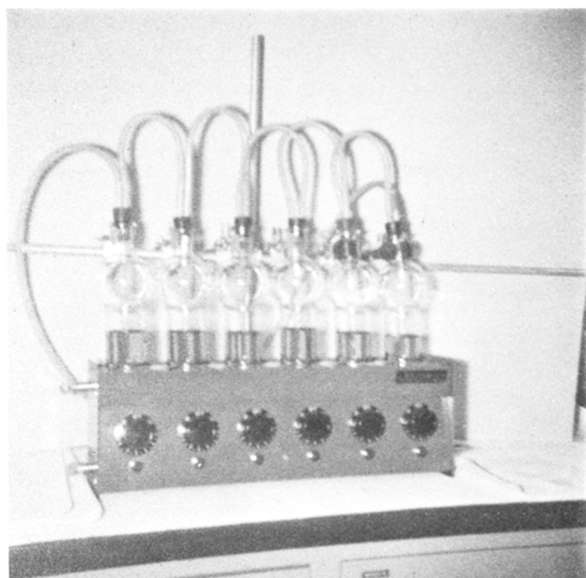

Figure 1. Reflux apparatus showing heating element, sample beakers, and condenser flasks.

hydrolysis and enzyme hydrolysis. When 67 or 80% of the mixture was hay, more D-glucose was produced by acid hydrolysis. This suggests that starch overestimation by acid hydrolysis is proportional to the cellulose concentration of the substrate. This is further verified by comparing the experimental values with calculated values (based on experimental starch values for alfalfa and grain separately).

Table IV shows the effects of the hydrolysis method on starch estimation in the feed and as the digesta moves through the gastrointestinal tract. Identical diets were fed to the two steers except that the sorghum grain was subjected to different processing. The grain in the diet fed to steer 1 was steamed and pressed into a flat flake (steam processed), whereas that fed to steer 2 was dry rolled as described by Hale et al. (1966).

Starch estimation was not affected by the hydrolysis method for feed or abomasal contents from either steer. Acid hydrolysis produced higher ($P < 0.01$) estimates of starch concentration in the small and large intestines and in the feces for both animals. Overestimation of starch due to the use of an acid-hydrolyzing medium ranged from 9% in the small intestine of steer 2 to nearly 300% in the feces of steer 1. By use of the feed and fecal starch values given in Table IV, enzyme hydrolysis results in a 98.2% starch digestibility in steer 1 vs. 93.2% for acid hydrolysis. Respective digestibility values for steer 2 would be 73.1 and 70.2%. Where starch content is high relative to cellulose content, errors resulting from acid hydrolysis are negligible. However, as the digesta moves through the digestive tract, starch is continually degraded and undigested cellulosic material becomes increasingly concentrated. This appears

Table V. Effect of Acid or Enzyme Hydrolysis on Estimation of Feed and Fecal Starch Content and Starch Digestibility[a]

item	acid hydrolysis	enzyme hydrolysis
starch in feed, %	65.3	66.3
starch in feces, %	9.4[b]	6.8[c]
starch digestion, %	96.8[b]	97.6[c]

[a] Composite data for 12 steers fed 80% sorghum grain diets (Kartchner, 1972). [b,c] Means on same line with unlike superscripts are different ($P < 0.01$).

to be the cause of the large differences in starch estimation by acid and enzyme hydrolysis as the digesta moves posteriorly. This effect appears to be greater where the starch is in a highly digestible form as in the diet fed to steer 1.

For determination of the effect of hydrolysis method on digestibility of high starch diets, the starch content of feed and feces from 12 steers fed 80% sorghum grain diets in a digestion trial (Kartchner, 1972) were analyzed by acid and enzyme hydrolysis and digestibility coefficients calculated on the basis of each analysis (Table V). The two methods gave similar dietary starch estimates, but acid hydrolysis estimated higher ($P < 0.01$) fecal starch content and, thus, a lower ($P < 0.01$) mean coefficient of starch digestibility than enzyme hydrolysis. However, absolute digestion coefficient values differed by less than 1 percentage unit (96.8 vs. 97.6%) between hydrolysis methods.

These data suggest that acid hydrolysis is acceptable for starch analysis in feeds with a starch content high in relation to cellulose. Under such conditions, acid hydrolysis gave results similar to those of enzyme hydrolysis. For fecal or digesta samples or feeds with a relatively high cellulose content, enzymatic hydrolysis would be the method of choice as starch content may be overestimated in the substrates if hydrolyzed by acid due to degradation of cellulose. Starch digestion by animals will likely be underestimated where acid hydrolysis is used, although differences appear small if high starch diets are utilized.

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LITERATURE CITED

AOAC "Official Methods of Analysis", 9th ed.; Association of Official Agricultural Chemists: Washington, DC, 1960; p 289, 22.043.

Frederick, H. M.; Theurer, B.; Hale, W. H. *J. Dairy Sci.* **1973**, *56*, 595.

Hale, W. H.; Cuitun, L.; Saba, W. J.; Taylor, B.; Theurer, B. *J. Anim. Sci.* **1966**, *25*, 392.

Kartchner, R. J. M.S. Thesis, University of Arizona, Tucson, AZ, 1972.

MacRae, J. C.; Armstrong, D. G. *J. Sci. Food Agric.* **1968**, *19*, 578.

Osman, H. F.; Theurer, B.; Hale, W. H.; Mehen, S. M. *J. Nutr.* **1970**, *100*, 1133.

Pazur, J. H.; Ando, T. *J. Biol. Chem.* 1959, *234*, 1966.
Steel, R. G. D.; Torrie, J. H. "Principles and Procedures of Statistics"; McGraw-Hill: New York, 1960; p 72.
Thivend, P.; Mercier, C.; Guilbot, A. In "Methods in Carbohydrate Chemistry"; Whistler, R. L., Ed.; Academic Press: New York, 1972; Vol. VI, pp 100–105.
Topps, J. H.; Kay, R. N. B. *Proc. Nutr. Soc.* 1969, *28*, 23A.

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Effects of Ultra-High-Temperature Pasteurization on Milk Proteins

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Ultra-high-temperature (UHT) pasteurization of skim milk (148 °C for 3 s) has been found to inactivate effectively foot and mouth disease virus. For determination of the effect of UHT pasteurization on milk proteins, the composition and properties of proteins from milk after this treatment were compared with those from conventional high-temperature–short-time pasteurized (HTST = 71.7 °C for 15 s) and raw skim milks. Vacuum-dried–acid-precipitated caseins and freeze-dried–dialyzed whey proteins were prepared from each product. Functional properties of casein such as solubility, viscosity, emulsifying capacity, and electrophoretic mobility were compared. For both casein and whey proteins, compositional comparisons were made among molar ratios of amino acids, total protein, and chemically available lysine. The solubility of milk caseins was reduced by UHT pasteurization. Whey protein nitrogen analyses show significant protein denaturation. No significant losses in nutritive value are indicated, and differences in viscosity and emulsification capacity are small.

Earlier studies (Burrows and Dawson, 1968; Hedger and Dawson, 1970; Sellers, 1969; Terbrüggen, 1932) indicated that the virus from foot and mouth disease (FMD) infected cows can survive in milk and derived milk products such as cheese (Blackwell, 1975) and casein (Cunliffe and Blackwell, 1977). FMD virus in whole milk, skim milk, and cream from experimentally infected dairy cows (Blackwell and Hyde, 1976) can survive conventional high-temperature–short-time (HTST = 71.7 °C for 15 s) pasteurization. Cunliffe et al. (1978, 1979) showed the persistence of infectious FMD virus up to 42 days in heat-dried caseins produced by acid precipitation of HTST-pasteurized skim milk from infected cows. Milk-borne FMD virus may pose a serious threat to FMD-free countries, and reliable procedures are needed to inactivate FMD virus in milk. Heat treatments considerably above pasteurization have been referred to as ultra-high-temperature (UHT) processes. According to the International Dairy Federation (1972), UHT processes refer to pasteurization techniques with temperatures of at least 130 °C in a continuous flow and holding times of ~1 s or more. Cunliffe et al. (1979) reported that UHT pasteurization effectively inactivates FMD virus in milk when carried out at 148 °C for 3 s or longer. It was of interest to determine the effect of this regimen on the properties of caseins and whey proteins prepared from UHT-pasteurized skim milk and to compare them with those prepared from HTST-pasteurized and raw skim milk. The results indicated that UHT pasteurization of skim milk resulted in interaction of casein and whey proteins, a reduction in solubility of casein at neutral pH or below, a decrease in chemically available lysine in whey protein, and a 56% denaturation of whey protein in the skim milk.

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EXPERIMENTAL SECTION

Sample Preparation. A schematic for the preparation of UHT-pasteurized, HTST-pasteurized, and raw caseins is shown in Figure 1. Fresh raw whole milk was obtained from a local dairy and separated cold. The raw skim milk, which contained 0.06% fat, was divided into three lots. Casein was prepared from 9.1 kg of lot no. 1 at 40 °C by precipitation at pH 4.6, with the addition of 1025 mL of 0.5 N HCl added slowly from a buret over a period of 15 min with stirring. After an additional 5 min of stirring, the casein settled and the whey was decanted and filtered. The casein was washed 4 times with water at pH 5.0. Water temperatures were 35, 45 (twice), and ~23 °C. Approximately 38.6 kg of lot no. 2 was pasteurized at 76.7 °C for 15 s in a triple tube heater, and the HTST-pasteurized casein was prepared as above from 9.1 kg of milk. Approximately 38.6 kg of lot no. 3 was sterilized in a tubular heat exchanger at 148.5 °C for 2.5 s, and 9.1 kg was used to prepare UHT casein as above except the skim milk was heated to 47.5 °C and 982 mL of 0.5 N HCl was added over a period of 7 min with stirring. Stirring was continued for an additional 10 min. The caseins from the raw, HTST-pasteurized, and UHT-pasteurized milks were dried in a shelf dryer under high vacuum.

Figure 2 shows the procedure for preparation of raw, HTST, and UHT freeze-dried whey proteins. Samples of all three wheys were dialyzed for 36 h in running chilled tap water and then for 12 h in distilled water. The dialyzed wheys were concentrated to approximately half of the

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