

How "Free" is "Gluten Free"? Relationship Between Kjeldahl Nitrogen Values and Gluten Protein Content for Wheat Starches

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ABSTRACT

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Kjeldahl nitrogen analysis of wheat starches submitted to three different laboratories showed poor precision. The relationship between mean total Kjeldahl protein ($N \times 5.7$) and gluten content was analyzed using a commercially available laboratory test kit based on specific antibodies. In general, the first 0.25% protein contained very little gluten, whereas most protein above the 0.3% level was in the form of gluten. Thus a

0.4% protein starch may contain up to 10 times as much gluten as a 0.3% protein starch. It was difficult using sodium-dodecyl sulfate polyacrylamide gel electrophoresis of starch extracts to clearly distinguish gluten polypeptides from starch granule proteins and to differentiate wheat starches that differed only slightly in protein content but markedly in gluten content.

Considering the simplicity (in chemical process terms) of wheat starch and gluten manufacture, the efficiency of separation of these fractions is quite high. However, it is well recognized that it is impossible industrially to remove all of the nitrogen-containing material from the starch—at least 0.25% protein ($N \times 5.7$) remains in the most pure starch, and it is not uncommon to find commercial wheat starch with 0.35–0.40% protein.

The proportions of different protein types within starch is relevant not only to understanding the structure and performance of starch in milling and its pasting properties, but also for the use of wheat starch in foodstuffs. It is important to minimize gluten content for use of wheat starch in baby foods, pharmaceuticals, and especially "gluten-free" baking mixes for celiac (gluten-intolerant) individuals (Cooke and Holmes 1984). The traditional method for protein determination of cereals and cereal products, including starches, is Kjeldahl nitrogen determination, wherein it is assumed that all nitrogen determined is present as protein, a reasonably accurate assumption in the case of wheat flour. In the case of wheat starch, a significant proportion of the nitrogen is nonprotein, being present in lipids such as lysophosphatidylcholine and phosphatidylcholine (Sulaiman and Morrison 1990). Furthermore, choline nitrogen in these lipids is less readily converted to ammonia than other sources of nitrogen, which could possibly cause variation in Kjeldahl results.

In the course of analysis of a wheat starch suspected of containing excessive gluten, we noted poor agreement between Kjeldahl results obtained for this starch between two different laboratories. This led us to analyze the precision of results obtained for a set of wheat starches in three laboratories and to determine the relationship between starch Kjeldahl data, protein content and composition, and actual gluten content. The results indicate that small differences in protein content of starches may be difficult to reliably detect by Kjeldahl analysis, although such differences can lead to relatively large differences in gluten content. This can have important implications for the suitability of the starch in critical applications, such as in gluten-free diets.

MATERIALS AND METHODS

Starch Samples and Kjeldahl Analysis

Seventeen wheat starch samples were obtained from each of three Australian manufacturers as well as from retail outlets. Each sample was blended thoroughly before analysis for gluten or

protein content. Each starch was analyzed by Kjeldahl nitrogen analysis using AACC Method 46-12 (AACC 1983) with the Kjeltec System (Tecator, Hoganas, Sweden). Several starch samples were submitted to either two or three industry laboratories with experience in Kjeldahl analysis of wheat starches. In some cases, the same laboratory analyzed the same starch sample (several times blind) at intervals of at least one week.

Gluten Analysis

The starch samples were also analyzed for gluten using a commercially available enzyme immunoassay kit (Medical Innovations Ltd., Artarmon, Australia). The kit method was developed by us (Skerritt and Hill 1990), and after international collaborative trials, received Association of Official Analytical Chemists First Action official method status (Skerritt and Hill 1991a) for gluten determination in foods. The antibodies used in this kit bind to certain ω -gliadin proteins found in similar proportions in grain of different wheat cultivars but do not bind in the assay to starch granule proteins (Greenwell and Schofield 1986). This method has a sensitivity of 0.0016% gluten in starch when a 1:5 dilution of the starch extract is used.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Replicate 4-g samples of nine of the starches, representing a range in protein contents, were extracted with 30 ml of 1% sodium dodecyl sulfate (SDS)-50 mM dithiothreitol for 4 hr at 50°C. The extracts were centrifuged and protein precipitated from the supernatants overnight at 4°C, using two volumes of acetone. The precipitates were redissolved in 100 μ l of 0.125M tris-HCl (pH 6.7)-2% SDS-5% 2-mercaptoethanol, and a 50- μ l aliquot was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) using a 12.5–15% gradient of polyacrylamide as previously described (Skerritt et al 1988). In a separate experiment, gliadin from five starches was extracted with 10 volumes of 40% ethanol by high-frequency homogenization (Ultraturrax, Janke and Kunkel, Dottingen, Germany). Extracts were clarified by centrifugation and the supernatants rotary-evaporated to dryness. The residue was redissolved in 500 μ l of SDS-mercaptoethanol and analyzed as above, except that a homogeneous (15% polyacrylamide) SDS-PAGE system was used. The former system has been optimized for the analysis of nongluten proteins associated with the starch granule and the latter for gliadins and glutenin polypeptides.

RESULTS AND DISCUSSION

Kjeldahl Nitrogen Analysis of Wheat Starches

In general, within-laboratory agreement (repeatability) was quite good (Table I), although some exceptions were found, such

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as laboratory 2 (sample f) and laboratory 3 (samples g and h). Laboratory 1 typically reported higher values than the others did. Some quite poor examples of between-laboratory precision were found, especially at lower mean protein values. At these values, protein contents are especially critical for the following reasons: 1) Codex recommendations (Codex 1981) imply that foods containing wheat starch with over 0.30% protein may not be labeled as gluten-free, and 2) premium food starches are often traded with maximum protein specifications required (e.g., 0.30 or 0.35%).

With the data available, it cannot be said that one laboratory gave somewhat poorer data than the others. General difficulties arise in starch nitrogen analysis, from the lower titration volumes used in the determination compared with flour or whole meal analysis. Use of more sample may counteract this problem, but poor digestion and loss of sample can then occur.

TABLE I
Precision of Starch Protein ($N \times 5.7$, %) Analysis
by Kjeldahl Nitrogen Determination

Starch Sample	Mean ^a	Laboratory			Inter-laboratory cv ^b
		1	2	3	
a	0.23	0.24	0.21	NT ^c	7
b	0.27	0.32	0.25	0.25	15
c	0.33	0.39, 0.35, 0.29	0.29, 0.28, 0.28, 0.29	0.37	12
d	0.33	0.32, 0.33	NT	NT	NT
e	0.36	0.40	0.31	NT	13
f	0.37	0.47	0.29, 0.39	0.29	24
g	0.41	0.42	0.40	0.52, 0.32	12
h	0.50	0.52	NT	0.40, 0.57	13
i	0.51	0.56	0.45	NT	9
j	0.54	0.53	NT	0.54	1

^aMeans of laboratory averages.

^bInterlaboratory coefficient of variance, calculated using the value obtained in the first analysis performed by the laboratory. The mean cv was 12% of the mean.

^cNT = not tested.

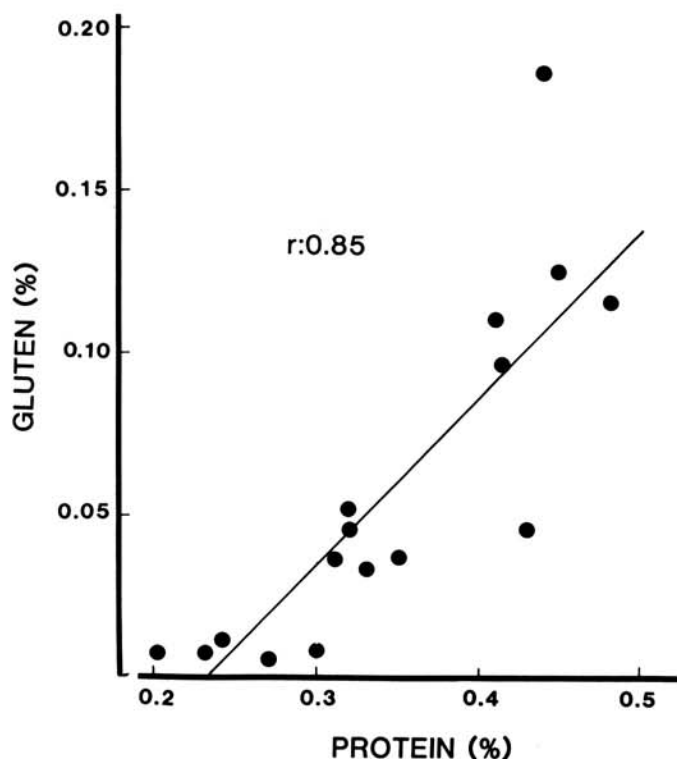


Fig. 1. Relationship between gluten content (determined by immunoassay) and Kjeldahl protein content ($N \times 5.7$) of commercial wheat starches.

Gluten Quantitation in Wheat Starches

Each of 19 starch samples was analyzed for gluten on two to five occasions using the laboratory test kit. Each wheat starch had detectable gluten ($>0.0016\%$), whereas no gluten was found in maize or potato starches analyzed at the same time. The relationship between starch protein content ($N \times 5.7$) and gluten content is shown in Fig. 1. It was linear ($r = 0.846$, $P < 0.0001$) with an equation: $\text{gluten (\%)} = -0.11 + 0.50 (\text{protein})$ over the narrow range studied. The "zero gluten" intercept was 0.23% protein. Therefore, these data suggest that approximately the "first" 0.25% of "protein" contains virtually no gluten, then both gluten and nongluten proteins increase, until above about 0.33%, virtually all extra protein in the starch is gluten. Thus the slope of the line steepens to equal near unity at higher protein concentrations (Skerritt and Hill 1990).

Much of the first 0.25% of protein in wheat starch consists of nongluten starch-granule proteins (see SDS-PAGE analysis described below) and nitrogen in lipids such as phosphatidyl choline (Sulaiman and Morrison 1990). The starch proteins, which may only account for about one third of the nitrogen in low-protein starch, have been characterized (Greenwell and Schofield 1986) and had only very weak homologies to gluten proteins (Skerritt et al 1990).

Analysis of Wheat Starches by SDS-PAGE

Nine starch samples, with varying nitrogen and gluten contents, were further analyzed by SDS-PAGE. Loadings on the gel were based on a constant mass of starch, so in general, those showing higher nitrogen contents had greater staining of polypeptide bands. As shown in Fig. 2, with the exception of starch 4 (purified A [large granule] starch, with very low protein, $N \times 5.7 = 0.20\%$), each starch had readily detectable bands at M_r 12,000, 19,000, 30,000, and 59,000, corresponding to major nongluten polypeptides identified earlier in highly pure, isolated starch (Greenwell and Schofield 1986). Other bands at M_r 5,000 and 8,000, corresponding to surface starch granule polypeptides (Skerritt

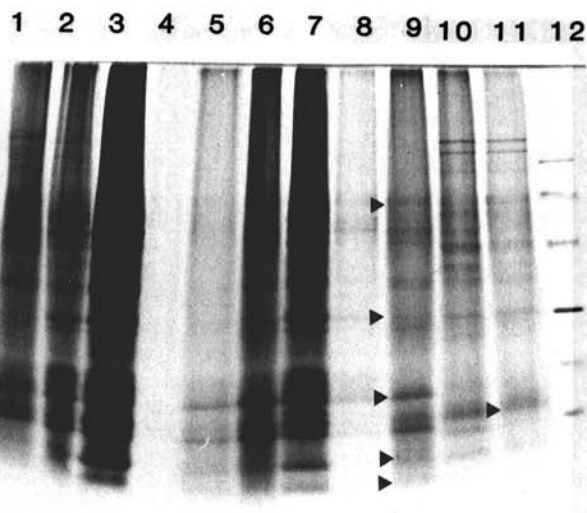


Fig. 2. Analysis of wheat starches by gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Molecular weight markers are shown. Starch granule polypeptides of molecular weights 5,000, 8,000, 19,000, 30,000, and 59,000, previously described (Greenwell and Schofield 1986), are indicated by arrowheads next to lane 9 and the polypeptide of molecular weight 15,000 by arrowhead next to lane 11. Starches (protein = $N \times 5.7$) are as follows: 1 = 0.29%, 2 = 0.33%, 3 = 0.43%, 4 = 0.20%, 5 = 0.23%, 6 = 0.43%, 7 = 0.41%, 8 = 0.28%, and 9 = 0.34%. Lanes 10 and 11 are extracts of two laboratory-washed soft wheat starches (Rosella variety, each about 0.40% protein). Lane 12 contains protein markers of molecular weights 14,400, 20,100, 30,000, 43,000, 68,000 and 92,000.

TABLE II
Gluten Content of Wheat-Starch-Based "Gluten-Free"
Baking Mixes^a

Manufacturer	Batch	Gluten (%, by immunoassay)	Acceptable? ^b
A	1988	0.25	No
	1989	0.018	Yes
B	1988	<0.016	Yes
	1989	0.052	No
C	1988	0.094	No
	1989	0.050	No
	1989	0.017	Yes

^aData shown are means of duplicate analyses.

^bAcceptable starches for a "gluten-free" diet contain less than 0.02% gluten (Skerritt and Hill 1991b).

et al 1990), were not found in the lowest protein starches, indicating that they can be washed off starch in commercial manufacture. Simultaneous analysis of starch from a pure sample of the soft wheat cultivar Rosella showed a polypeptide of *Mr* 15,000, which has been shown to be a marker of endosperm softness. This band was faint or absent in most commercial starches, since in Australia, hard wheat is often used for starch-gluten manufacture.

The higher-protein starches had a number of polypeptides above *M* 30,000; these likely represent gliadin and glutenin polypeptides. Analysis of 40% ethanol extracts of two low- and two high-protein starches (0.25 and 0.5%, respectively) revealed detectable protein in the gliadin molecular weight range in only the high protein starches (data not shown). Therefore, while SDS-PAGE could distinguish starches that were very high in protein (and thus high in gluten) from starches that were very low in protein, it was not suitable for discriminating starches with protein contents around the critical value of 0.30%, above which gluten content can become significant. For example, starches 1, 5, 8, and 9 had similar profiles (Fig. 2), although their protein contents varied from 0.23 to 0.34%, and their gluten contents varied much more, from <0.01 to 0.05%.

Analysis of Gluten-Free Mixes with Wheat Starch Base

To investigate whether variation in wheat starch quality was occurring in the manufacture of gluten-free foods, samples of gluten-free baking mixes based on wheat starch were obtained from three manufacturers over two years. Significant lot-to-lot variation of the same product was noted in gluten content (Table II), with about half of this set of batches having unacceptably high gluten contents. The two gluten products highest in gluten had been referred to us by local celiac societies after suspected adverse reactions in members. Kjeldahl analysis of these products would have had little meaning due to the presence of other proteins from milk and yeast and sometimes maize, soy, and buckwheat.

CONCLUSIONS

Kjeldahl nitrogen analysis of starches was found to be rather inaccurate and imprecise, especially around the 0.30% protein specification for gluten-free foods or the 0.35% protein sometimes specified in contracts for the purchase of A-grade wheat starch. Similarly, SDS-PAGE can only detect large differences in protein content of starches. On average, the first 0.25% of protein in wheat starch is associated with nongluten starch granule protein and nitrogen-containing phospholipid. Extra protein is largely gluten contamination, such that slight increases in protein content can lead to disproportionately larger differences in gluten content. Gluten-free baked foods based on wheat starch lack the taste, aroma, and textural properties of foods based on flour starch. While maize, potato, or rice starches could be used, many gluten-intolerant individuals and speciality food manufacturers feel that

given the role of wheat starch in baking (Dennett and Sterling 1979), it is a superior base for these foods.

Provided that it is well washed, wheat starch is a good base for gluten-free baked foods (Skerritt et al 1987). Controlled trials (Ejderhamn et al 1988) have shown no evidence of ill effects in celiac individuals after long-term wheat starch consumption. Finally, starch granule proteins did not display glutenlike activity on blood lymphocytes of celiacs (Penttila et al 1991).

Nevertheless, it is important to minimize the content of gluten in the diets of celiacs, not only because moderate amounts may cause immediate diarrhea and vomiting, but because ongoing consumption of small amounts of gluten may be associated with increased risks of malignancy (Holmes et al 1989). Batch-by-batch testing is therefore critical for wheat starches intended for special dietary foods. For this purpose, gluten may be specifically quantitated using a simple commercially-available laboratory test of high precision (Skerritt and Hill 1991a) or by rapid screening with a 7-min test (Skerritt and Hill 1991b).

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