

CHARACTERISTICS OF HIPROLY BARLEY II. QUANTIFICATION OF TWO PROTEINS CONTRIBUTING TO ITS HIGH LYSINE CONTENT

by

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Quantitative extraction of salt and water soluble proteins from Hiproly, CI 4362 and Bomi barley was performed and their content of the lysine rich SP II albumin determined by a single radial immunodiffusion assay. The elevated content of SP II albumin in Hiproly accounted for 37% of the difference in crude protein lysine between Hiproly and Bomi and for 19% between Hiproly and its low lysine sisterline CI 4362. Apart from the lysine rich SP II albumin, other proteins contribute to the high lysine content of Hiproly.

The single radial immunodiffusion assay for quantitative determination of SP II albumin permits the separation of high-lysine from low-lysine lines in progenies from crosses with Hiproly. Identification of plants carrying the Hiproly gene can be carried out by single seed analysis and is more reliable than identification by β -amylase content or by dye-binding capacity measurements. Degree of grain hardness as measured by energy required for milling can vary independently of the content of SP II albumin.

1. INTRODUCTION

Studies of the albumin proteins extracted from Hiproly grains identified two lysine rich albumin polypeptides (14). They have been designated SP II A and SP II B and were also found to be notably rich in valine, glutamine/glutamic acid

and asparagine/aspartic acid. Immunologically the two proteins are very similar if not identical and therefore the SP II B polypeptide with a molecular weight of 8,100 dalton is considered a fragment of the SP II A peptide with a molecular weight of 9,100 dalton.

In this paper a quantitative analysis of the amount of the two lysine rich albumin proteins in the grains of Bomi and Hiproly barley and in those of Hiproly's low-lysine sisterline CI 4362 is presented. This permits decision as to whether the high-lysine content of Hiproly is due to a high content of these two proteins. In addition various high- and low-lysine barley lines were analysed in order to study to what extent the lysine content is correlated with the amount of the two lysine rich albumin proteins. A radial immunodiffusion assay was developed for the quantitative determination of the SP II A and SP II B proteins. This allows to screen for the amount of these two proteins on a single kernel basis.

2. MATERIALS AND METHODS

2.1. Plant material

The 36 barley lines examined (Table I) included 14 commercial barley varieties grown at Carlsberg's Research Farm, Allindemaglegård, in 1976. The residual 22 lines were part of Carlsberg Plant Breeding's high-lysine barley nursery. Hiproly and CI 4362 were harvested in 1978 at Carlsberg's Research Farm, Hyldager-

gård. 6 lines originated from the Swedish Seed Association, Svalöf, Sweden, and consisted of homozygous lines from the crosses listed in Table I. These lines were grown at Hyldagergård in 1976. The residual 14 lines were grown at Hyldagergård and comprised repeated backcrosses of different commercial barley varieties with Hiproly. 12 of the lines were grown in 1975 and 2 lines in 1976.

2.2. Extraction of the water- and salt-soluble protein fraction of the grain

Barley flour was obtained by grinding in a Cyclotec sample mill (Udy Analyser Company, Boulder, Colorado, USA) using a 1 mm screen.

2.2.1. Extraction procedure for screening

Duplicate samples of barley flour (2 g) were extracted for 1 hour at room temperature by shaking (Rotamix, Heto, Birkerød, Denmark) with 20 ml of a 0.5 M-NaCl solution in a 29 mm diameter, round bottomed centrifuge tubes. The extracts were clarified by centrifugation ($27.000 \times g$ for 25 min), and the volume of each extract was measured after decantation. Samples from

Table I

List of barley lines examined.

Low-lysine lines	High-lysine lines
1. Athos	20. Hiproly
2. Goldfield	21.-23. [Hiproly \times (Pallas ⁵ \times Glacier)] \times [Å6157 \times (Mari ⁵ \times Tr.a.l.)]
3. Welam	24. (Bomi ² \times Hiproly) \times Elbo
4. Zita	25.-29. Minerva ² \times (Bomi ² \times Hiproly)
5. Menuet	30.-31. (Bomi ² \times Hiproly) \times Cilla ²
6. Mirjam	32. Elbo ² \times (Bomi ² \times Hiproly)
7. Foma	33.-34. Cilla ² \times (Bomi ² \times Hiproly)
8. Nordal	35. Bomi ² \times Hiproly
9. Alf	36. Gerkra \times (Bomi ² \times Hiproly)
10. Aura	
11. Emir	
12. Ohra	
13. Lofa	
14. Bomi	
15. CI 4362	
16.-18. [Hiproly \times (Pallas ⁵ \times Glacier)] \times [Å6157 \times (Mari ⁵ \times Tr.a.l.)]	
19. (Bomi ² \times Hiproly) \times Nordal ²	

each extract were taken for further analysis, and the residual extracts dried in an oven at 110 °C for Kjeldahl determinations.

2.2.2. Quantitative extraction of the water- and salt-soluble protein of barley

Four successive extractions (12) of Hiproly, CI 4362 and Bomi were carried out as described in section 2.2.1. Samples for analysis were taken after each round of extraction. The four flour extracts from each barley line were pooled and dried in an oven at 110 °C for Kjeldahl analysis.

2.2.3. Extraction procedure for single seed analysis

The extraction method applied was essentially the one described by AASTRUP (1). Kernels were ground individually in a mill, and the flour was weighed into plastic tubes. The flour was suspended in 0.5 M-NaCl solution (0.01 ml per mg flour), and the tubes were shaken for one hour. The flour was removed by centrifugation, and 0.005 ml samples were taken for analysis.

2.3. Amino acid analysis

Amino acid analysis was performed both on crude- and salt-soluble barley protein. All

samples were analysed on a Durrum D-500 amino acid analyser. Cysteine and tryptophane were not determined.

2.3.1. Amino acid analysis of the crude barley protein

Duplicate samples of 50 mg accurately weighed barley flour were hydrolysed in 5 ml 6 N-HCl, 0.05% w/v phenol and 0.001 M-norleucine for 24 hours at 110 °C in evacuated ampoules. Values for the amino acid composition of the crude protein are expressed as g amino acid per 100 g protein (Kjeldahl N × 6.25), and the average of double determinations is given.

2.3.2. Amino acid analysis of the water- and salt-soluble protein of barley

Duplicate samples of 0.050 ml salt extract (section 2.2.1) were lyophilized and hydrolysed in 0.200 ml 6 N HCl containing 0.05% w/v phenol for 24 hours at 110 °C in evacuated ampoules.

2.4. Quantification of SP II albumin by single radial immunodiffusion

Rabbit antisera, raised against the two high-lysine albumin proteins of Hiproly barley (SP II

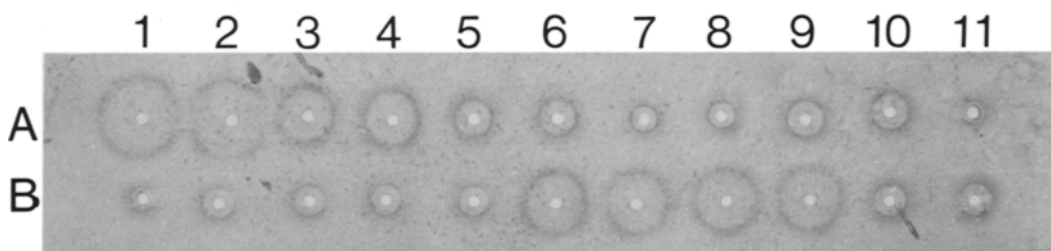


Figure 1. Quantification of SP II albumin by means of radial immunodiffusion.

The area of the circles is directly proportional to the amount of SP II albumin applied in the sample well (5 µl).

Key to wells:

2.05 µg SP II albumin A 1,2

1.03 µg SP II albumin A 3,4

0.53 µg SP II albumin A 5,6

0.26 µg SP II albumin A 7,8

Crude extract of Hiproly B 6-9

Crude extract of CI 4362 B 2-5

Crude extract of low-lysine lines A 11, B 1

Crude extract of high-lysine lines from crosses with Hiproly A 9, 10, B 10, 11

A and SP II B protein) as described by JONASSEN (14), were used in this study. The method of single radial immunodiffusion by MANCINI et al. (15) was modified as follows: A 1% w/v buffered agar solution was obtained by boiling 1 g of agar (type HSA from Litex, Postbox 7, 2600 Glostrup, Denmark) in tris-barbital buffer, pH 8.6 (0.073 M-tris, 0.0245 M-barbital, 0.00036 M-calciumlactate, pH 8.6). The agar solution was cooled down to 57 °C in a water bath, and 0.6 ml of a 10 times concentrated and purified antiserum were mixed 33 ml agar solution and poured in a mould (dimensions, 18.4 × 10 × 0.15 cm). 55 antigen wells (diameter 2.1 mm) were punched into each gel plate, and 0.005 ml samples applied (Figure 1). Each gel plate included 4 duplicate standards of purified antigen. All radial immunodiffusion plates were stained by Coomassie Brilliant Blue R 250 (4). The amount of albumin was determined with a calibration curve using known amounts of SP II albumin.

2.5. Quantification of β -amylase by rocket immunoelectrophoresis

Rabbit serum against the salt-soluble extract (albumin and globulin) of barley was obtained and purified as described by HARBOE and INGILD (7).

0.005 ml diluted samples of barley salt extracts (section 2.2.1) were subjected to rocket immunoelectrophoresis as described by AXELSEN et al. (4). The gels were stained for β -amylase activity after electrophoresis by the starch-iodine method (8). The β -amylase content of the different samples was determined by a standard curve obtained from solutions of purified barley β -amylase.

2.6. Determination of milling energy

5 g whole grain of each barley sample were ground in a mill, and the required energy was recorded. The method employed was a modified version of the one described by ALLISON et al. (2): 5 g samples were ground by an Alpine Perplex 100 LU mill (Alpine AG, D-89 Augsburg 1, Germany) mounted with a 2 mm screen. The electric energy required was recorded by a power to voltage converter connected to the mill. The total energy required to mill a sample was estimated by integrating the area of the trace from the recorder chart. The integrations were carried out with a Hewlett Packard digitizer (HP 9864 A) connected to a Hewlett Packard calculator (HP 9825 A).

Table II

Protein content (Kjeldahl N × 6.25) and lysine content of protein fractions in three barley varieties.

Variety	Hiproly CI 4362 Bomi			Hiproly CI 4362 Bomi			Hiproly CI 4362 Bomi		
Samples	Total protein			Salt-soluble protein			SP II albumin		
Protein, g per 100 g dry matter of seeds	17.3	15.3	11.7	4.77	3.85	2.94	0.284	0.077	0.012
Lysine mg per 100 g dry matter of seeds	650	468	383	266	176	148	33.6	9.1	1.4
g lysine per 100 g protein	3.76	3.06	3.27	5.59	4.59	5.05	11.84 ^{a)}		
Lysine, % of lysine in the seed				40.9	37.6	38.6	5.2	1.9	0.3
SP II lysine, % of salt-soluble lysine							12.6	5.1	0.9

a) The figure is calculated from the amount of SP II A and SP II B in Hiproly (14).

2.7. Determination of the starch-protein adherence character

10 kernels of each barley line were classified for endosperm type under the microscope, as described by MUNCK et al. (18).

2.8. Water determination of barley flour

The water content of barley flour samples was measured as the decrease in weight after 1 hour in an oven at 130 °C.

2.9. Determination of protein content

Protein content of barley flour and dried protein extracts (section 2.2) were determined by the Kjeldahl-method using the Tecator System (Tecator, Höganäs, Sweden) with a selenium catalyst (Kjeltabs, Bie and Berntsen, Copenhagen, Denmark). Protein is expressed as Kjeldahl N \times 6.25.

2.10. Determination of dye-binding capacity

Duplicate samples of barley flour (0.5 g) were determined for dye-binding capacity according to the Udy-method (21) using the Tecator System (Tecator, Höganäs, Sweden).

3. RESULTS AND DISCUSSION

3.1. Quantification of SP II albumin in Hiproly, CI 4362, and Bomi barley

The seed proteins of Hiproly, CI 4362, and Bomi barley were quantitatively extracted in order to determine how much the content of SP II albumin contributes to the amino acid composition of the crude- and salt-soluble protein fraction of the three barley lines. Protein was calculated as Kjeldahl N \times 6.25. The SP II protein was determined by radial immunodiffusion section 2.4) and calculated as Kjeldahl protein (N \times 6.25) from the molecular weight and amino acid content (14). It can be calculated from the data in Table II that Hiproly contains 24 times more SP II albumin on a dry matter basis than the commercial barley variety Bomi. The sisterline CI 4362 is intermediate between Bomi and Hiproly, containing 6.4 times more SP II albumin than Bomi and 3.6 times less than Hiproly. The lysine content per 100 g crude

protein of the three barley lines is listed in Table II, and it can be calculated that the elevated content of SP II albumin in Hiproly accounted for 37% of the difference in lysine content between Bomi and Hiproly and for 19% between CI 4362 and Hiproly. According to Table II the salt-soluble protein of Hiproly contains 5.59 g lysine per 100 g protein. This figure includes a contribution of 0.70 g lysine from the SP II albumin. The figure for Bomi is 5.05 g lysine per 100 g protein and includes a contribution of only 0.05 g lysine from the SP II albumin. The differences in the lysine content of the salt-soluble proteins among the three varieties are very well correlated with the differences in the lysine content of the total protein. On the other hand, when the difference in lysine content between Hiproly and Bomi are compared with the lysine contribution from the SP II albumins, it is clear that other differences in the composition of the salt-soluble protein must be present among the three lines besides the difference in SP II albumin content. Multiple differences between Hiproly lines and low-lysine barley have been

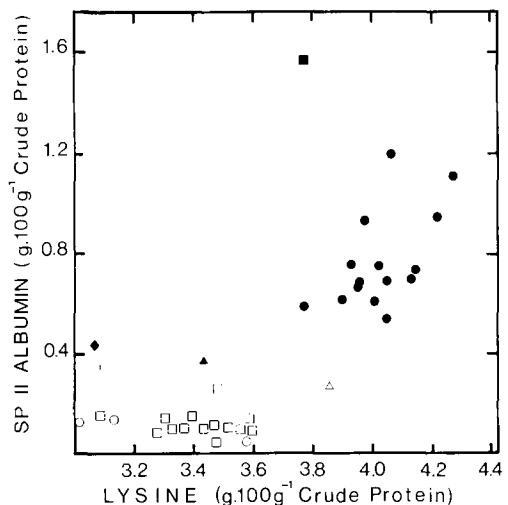


Figure 2. Plot of amount of SP II albumin per 100 g crude protein versus amount of lysine per 100 g crude protein in different barley lines.

■ Hiproly, ◆ CI 4362, □ commercial barley varieties, ● high-lysine lines from crosses with Hiproly, ○ low-lysine lines from crosses with Hiproly, ▲ line 17, Table I, and △ line 36, Table I with plants displaying intermediate values with respect to lysine and SP II albumin content.

demonstrated in the case of β -amylase (3) and by several electrophoretic studies (11, 13, 17). It was pointed out by HAGBERG et al. (6) that the CI 4362, of Ethiopian origin, was morphologically very similar to Hiproly, but that its amino acid composition was very close to that of conventional barley. It was therefore expected that Hiproly and CI 4362 displayed a simple relationship with respect to lysine content, for example that the reduction of SP II albumin in CI 4362 could explain the difference in lysine content. However, CI 4362 contained more SP II albumin than would have been expected from its overall lysine content (Figure 2).

It should be noted that the extractions performed are only quantitative with respect to SP II albumin soluble in 0.05 M-NaCl solution, and it is possible that residual SP II albumins are bound in the insoluble residue. This question needs further investigation. Determination of SP II albumin by means of single radial immunodiffusion has advantages and limitations. The major advantage is its simplicity, low costs, and the ease with which the method can be applied to mass screening. A major limitation is due to the fact that it becomes increasingly difficult to measure with accuracy the diameter of the precipitate as it diminishes and this will tend to overestimate small precipitates (cf. Figure 1).

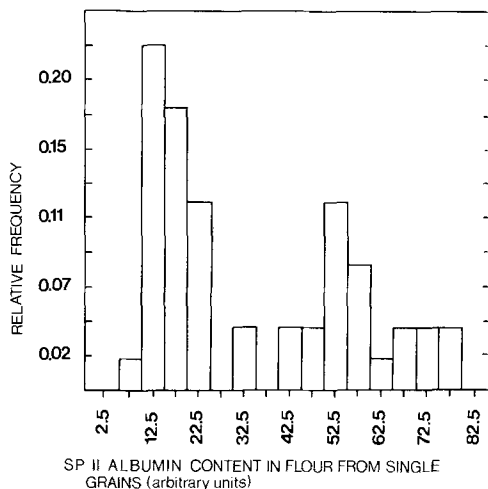


Figure 3. The histogram displays the distribution of the SP II albumin content in 50 seeds from line 17 (Table I) (Figure 2, ▲) derived from a cross with Hiproly.

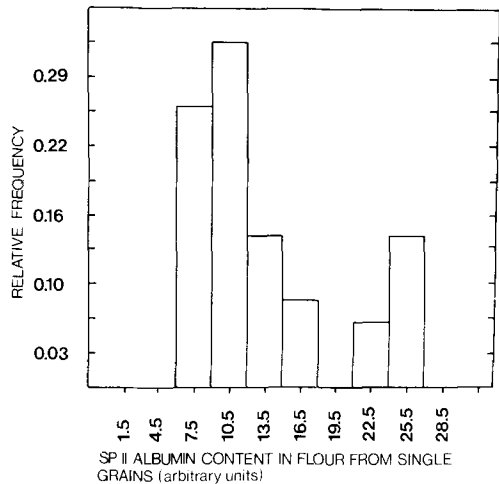


Figure 4. The histogram displays the distribution of the SP II albumin content in 50 seeds from line 36 (Table I) (Figure 2, △) derived from a cross with Hiproly.

3.2. Contents of SP II albumin in different barley lines

In order to determine if the content of SP II albumin is diagnostic for the Hiproly character in many different genetic backgrounds 22 experimental barley lines and 14 commercial varieties were analysed. The barley material was extracted as described in section 2.2.1, and the content of SP II albumin determined by radial immunodiffusion. In Figure 2 the SP II albumin content per 100 g crude protein of the lines is plotted against their lysine content per 100 g crude protein. This results in two discrete groups of barley lines. One group is high in lysine (about 4 g lysine per 100 g crude protein) and contains more than 0.54 g SP II albumin per 100 g crude protein, whereas the other group is low in lysine (about 3.4 g lysine per 100 g crude protein) and contains less than 0.25 g SP II albumin per 100 g crude protein.

It can be seen that both CI 4362 and Hiproly fall outside these two groups, in fact CI 4362 has a considerable amount of SP II albumin but very little lysine, whereas Hiproly has more SP II albumin than any other investigated line. Two lines derived from crosses with Hiproly fall outside the two groups described above as they have an intermediate lysine and SP II albumin

content. To investigate the nature of the two lines further, 50 seeds of each line were individually extracted (section 2.2.3.), and the content of SP II albumin per mg seed was determined. The histograms in Figures 3 and 4 show that the two barley lines contained seeds differing significantly in SP II albumin content. The histogram in Figure 3 reveals separated

classes of seeds, one class having a high content of SP II albumin and the other a low one. The number of individuals in each class is approximately the same, and since this line has been propagated in bulk for 6 generations it is likely that it was started from a plant heterozygous for the lys gene. The analysis of this line demonstrates that screening for SP II albumin with the

Table III

Characteristics of barley lines with a low content of SP II albumin versus lines high in SP II albumin.

Analysed character	Mean and standard deviation for 17 lines containing less than 0.25 g SP II albumin per 100 g crude protein	Mean and standard deviation for 15 lines containing more than 0.54 g SP II albumin per 100 g crude protein	$\bar{X}-\bar{Y}$ t-test
	\bar{X}	\bar{Y}	
g SP II per 100 g crude protein	0.108 ± 0.012	0.708 ± 0.050	-0.600***
g lys per 100 g crude protein	3.38 ± 0.04	4.02 ± 0.03	-0.64 ***
g lys per 100 g salt protein	4.95 ± 0.06	5.57 ± 0.11	-0.62 ***
g val per 100 g crude protein	5.03 ± 0.05	5.28 ± 0.03	-0.25 ***
g val per 100 g salt protein	4.99 ± 0.05	5.78 ± 0.11	-0.79 ***
g arg per 100 g crude protein	4.94 ± 0.07	5.13 ± 0.06	-0.19 *
g arg per 100 g salt protein	6.65 ± 0.09	6.31 ± 0.13	0.34 *
g crude protein per 100 g dry matter	12.30 ± 0.22	13.82 ± 0.46	
g salt protein per 100 g dry matter	2.47 ± 0.04	3.05 ± 0.10	
milling energy, cm ²	12.86 ± 0.60	16.45 ± 0.53	
g β-amylase per 100 g salt protein	0.692 ± 0.076	1.560 ± 0.127	

***: significance at the 0.01% level, *: significance at the 5% level. Salt protein: protein soluble in 0.5 M-NaCl and expressed as g N × 6.25. Milling energy is expressed as trace area required to grind 5 g of barley seeds.

immunodiffusion method on single seeds is ideal to select high-lysine segregants in Hiproly crosses.

In spite of the promising results in improving yield of Hiproly genotypes (6), no high yielding high-lysine barley has as yet become available (5). The yield level is restricted by small seed size combined with poor fertility (19). Furthermore, the backcross lines seem to be more sensitive to extreme weather conditions than many high yielding commercial varieties (19). In selecting high-lysine segregants from Hiproly crosses, it appears to be difficult to combine the high-lysine content (g per 100 g protein) with high yield. It will be possible to test this on Hiproly lines with the immunodiffusion assay by rapidly selecting a large number of lines with a high content of this protein.

3.3. Evaluation of the SP II albumin screening method

The two lines (Figure 3 and 4) which turned out to be populations of two different SP II albumin genotypes were excluded from this part

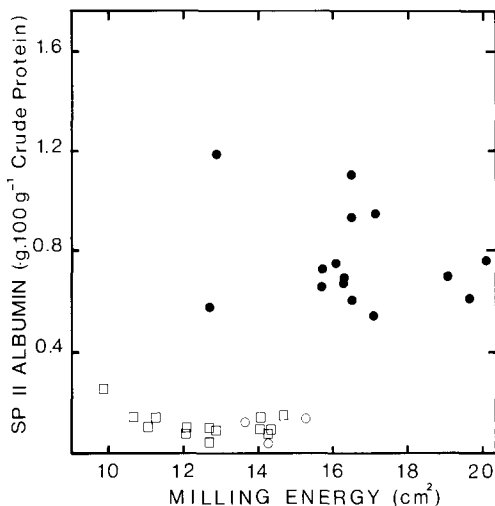


Figure 5. Plot of milling energy versus amount of SP II albumin per 100 g crude protein.

● High-lysine lines (Table I, 21–35) from crosses with Hiproly. ○ Low-lysine lines (Table I, 16, 18, 19) from crosses with Hiproly. □ Commercial barley varieties (Table I–14).

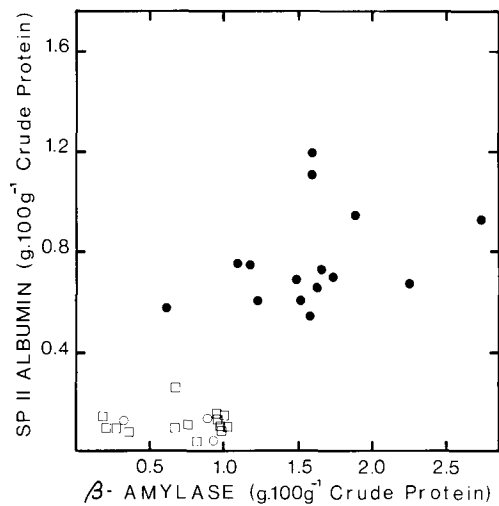


Figure 6. Plot of amounts of β -amylase per 100 g crude protein versus amount of SP II albumin per 100 g crude protein.

● High-lysine lines (Table I, 21–35) from crosses with Hiproly. ○ Low-lysine lines (Table I, 16, 18, 19) from crosses with Hiproly. □ Commercial barley varieties (Table I–14).

of the work as well as Hiproly and CI 4362 and it was deduced from Figure 2 that the remaining high-lysine lines within the given limits were homogeneous with respect to their SP II albumin content. The amount of SP II albumin (g per 100 g crude protein or salt-soluble protein) was calculated for the remaining lines. The data are presented in Table III. It has been shown that the elevated content of lysine in Hiproly is significantly associated with an increased content of valine and arginine (20). The SP II albumin contains relative large amounts of lysine and valine, (14), and it can therefore be expected that the SP II albumin contributes significantly to the increased content of these amino acids in the high-lysine segregants compared to low-lysine segregants in crosses. The t-test (Table III) shows that the high valine content of the high-lysine lines is significantly associated with a high content of SP II albumin (significance at the 0.01% level), not only in the salt-soluble protein, but also in the crude protein. On the other hand the increased arginine content of the high-lysine lines cannot be due to a high content

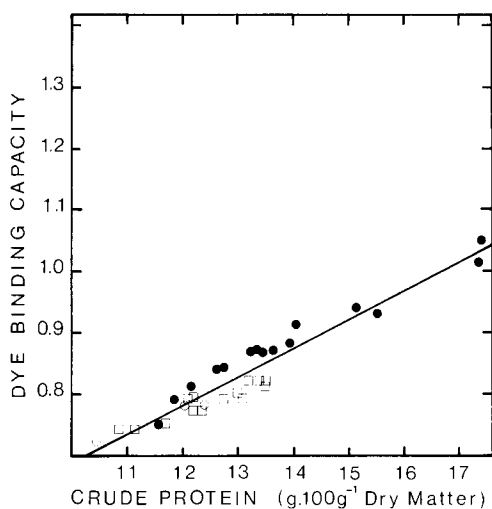


Figure 7. Plot of amounts of crude protein per 100 g dry matter versus dye-binding capacity.

Crude protein is determined as Kjeldahl N \times 6.25. Dye-binding was measured by the Udy-method (section 2.10). The line of regression is calculated from all points. ● High-lysine lines (Table I, 21–35) from crosses with Hiproly. ○ Low-lysine (Table I, 16, 18, 19) from crosses with Hiproly. □ Commercial barley varieties (Table 1–14).

of SP II albumin as its content of arginine is not specifically high.

3.4. Comparisons between the SP II albumin screening method and other screening methods for the Hiproly character

Starch-protein adherence has been used as a marker for the Hiproly character (10, 18). To investigate if there is a connection between the starch-binding factor and the amount of SP II albumin, 10 seeds from each line were examined for the starch-protein adherence character. All lines containing more than 0.54 g SP II albumin per 100 g crude protein displayed an intermediate starch-binding character when compared to Hiproly barley lines low in SP II albumin. It was not possible to demonstrate a close connection between starch-linking and high amount of SP II albumin content because of the intermediate nature of all high-lysine lines tested. MUNCK (17) has suggested that there might be a connection between the Hiproly character and grain hard-

ness. The groups from the starch-binding determinations acted homogeneously when determined for milling energy (Figure 5). However, two lines with more than 0.54 g SP II albumin per 100 g crude protein had milling energies comparable to the lines low in SP II albumin (less than 0.25 g SP II albumin per 100 g crude protein). This indicates that an elevated SP II albumin content does not directly affect grain hardness measured as milling energy.

Hiproly contains large amounts of soluble β -amylase compared to commercial barley varieties (3) and segregants from Hiproly crosses low in lysine. The screening method of HEJGAARD et al. (9) was used as described in section 2.5, and β -amylase content was measured and plotted versus SP II albumin content (Figure 6). The β -amylase quantification method does not give a clear cut separation between the two groups being high and low in SP II albumin content. It should be noted that an elevated β -amylase content tends to reduce the overall lysine content of the seeds because of the relatively low lysine content in β -amylase itself (3.9 mole %).

Dye-binding capacity versus crude protein in dry matter (Figure 7) has been recommended by MOSSBERG (16) as a screening method for high-lysine cereals. It is shown in Figure 7 that the line of regression does not produce a sharp separation between the two SP II albumin groups. This result is not unexpected because the method measures the amount of basic amino acids (16). Determination of the SP II albumin content has given the best separation of the low- from the high-lysine lines containing the Hiproly character.

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