SEGREGATION OF HIGH AND LOW CHYMOTRYPSIN INHIBITOR CI-2 CONTENT IN CROSSES BETWEEN HIPROLY AND NORMAL LYSINE BARLEY GENOTYPES

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Keywords: Serine proteinase, subtilisin, maternal inheritance, digestibility

Half barley seeds derived from reciprocal crosses between Hiproly and the normal lysine genotypes Nordal and Welam as well as between Hiproly and CI 4362 (Hiproly isogenic sisterline) were analyzed for their content of the lysine rich chymotrypsin inhibitor named CI-2. Analysis of F_1 and F_2 seeds suggested that a high content of inhibitor CI-2 is inherited as a single recessive trait and confirmed the close genetic relationship between Hiproly and CI 4362. Expression of a high CI-2 content in F_1 was found to be different in reciprocal crosses of Hiproly with Nordal or Welam. Screening for Hiproly segregants by dye binding was compared to analysis by the CI-2 quantification method. A good correlation was obtained and this permits the conclusion that a high CI-2 content is diagnostic for the presence of the *lys* gene. Quantification of CI-2 in half seeds is useful for breeding towards an increase in this lysine-rich protein.

1. INTRODUCTION

The high lysine content of Hiproly barley seed protein is due to a slight increase in its salt soluble protein fraction (albumin and globulin) and to an increase in the lysine content of this fraction compared to normal lysine barley genotypes (2,12,13). Extensive fractionation of the Hiproly albumin fraction revealed that the content of one protein of MW 9.100 containing 11.5 g lysine · 16 g N⁻¹ was greatly increased compared to its content

in normal lysine barleys (6,7). The isolated protein was named SP II albumin in accordance with the chromatographic fraction from which it was obtained. Later investigations revealed, that the protein showed proteinase inhibitor activity towards chymotrypsin and subtilisin, and that it displayed active site and amino acid sequence homology with the chymotrypsin inhibitors of the potato inhibitor I family (8,18,20). This new knowledge made it appropriate to rename the

inhibitor chymotrypsin inhibitor 2 (CI-2), in agreement with its activity and relationship to the potato inhibitor I family (11). Hiproly also contains increased levels of another chymotrypsin inhibitor named CI-1 (5). It has been suggested that proteolytic inhibitors in cereals may limit the utilization of cereal diets (19), and such effects have been studied with the trypsin inhibitors of Triticale (3). The two inhibitors (CI-1 and CI-2) which contribute prominently to the high lysine content of Hiproly (5,7) have proved to be very susceptible to degradation in the acidic peptic environment of monogastric animals (1). This may explain why Hiproly displays a greatly improved lysine and protein digestibility compared to normal lysine barley varieties (10,14).

The high lysine character of Hiproly was originally identified using the dye binding capacity (DBC) method and KJELDAHL determinations by MUNCK et al. (14). Analysis of the offspring from crosses between Hiproly and normal lysine barley genotypes based on histological and chemical analyses suggested that the high lysine character was determined by a single recessive gene named *lys* (14). Later investigations showed that high lysine lines derived from crosses with Hiproly all contained a high level of inhibitor CI-2 although not reaching the level of Hiproly itself (7).

The purpose of the present communication is to present some data on the inheritance of the content of CI-2 in progenies from crosses involving Hiproly and to examine the relationship between the contents of CI-2 and the amount of lysine as determined by the DBC method. The use of the two analytical methods in Hiproly barley breeding is discussed.

2. MATERIALS AND METHODS

2.1. Plant material

Seeds of Hiproly, CI 4362 (Hiproly sisterline with a low lysine content) and the normal lysine genotypes Nordal and Welam were grown in a green house in the summer of 1980 and reciprocal crosses were made between Hiproly and the three genotypes. It is well established that Hiproly is difficult to maintain as a pure line due to cross-pollination. The Hiproly material used in this study was selected from plants with typical Hiproly seed characteristics. Parents were manually selfed as controls. F_1 seeds were planted in

September 1980 and F_2 seeds were harvested for half-seed quantification of chymotrypsin inhibitor CI-2. Half F_2 seeds, covering the entire variation in CI-2 content (see section 2.2) from the reciprocal crosses between Hiproly and Nordal were sown in the Phytotron at the Swedish University of Agricultural Science, Stockholm, in the spring of 1981, and plants were harvested individually. The plants were grown together and not isolated.

2.2. Quantification of inhibitor CI-2 in half seeds by radial immunodiffusion.

Seeds from the F_1 , F_2 and F_3 generation were dissected transversely with a scalpel. The distal part was used for analysis and the embryo part saved for propagation. Half-seeds were crushed with an electric drill, weighed into Eppendorf tubes and suspended in $10 \mu\text{l} \cdot \text{mg seed}^{-1}$ of extraction buffer containing 0.5 M-NaCl, 0.8 M-urea and 0.2% Triton X-100. After two days incubation at room temperature the samples were shaken vigorously for one hour and clarified by centrifugation. $5 \mu\text{l}$ supernatant was used for radial immunodiffusion. Preparation of monospecific antisera towards inhibitor CI-2 and immuno-diffusion gels have been described earlier (6,7).

Melted agar 57°C containing $65 \mu\text{l serum} \cdot \text{ml}^{-1}$, 15 mM- NaN_3 and buffered with Tris/barbital at pH 8.6 was cast onto a glass-plate using a mould ($20 \times 10.2 \times 0.1 \text{ cm}$). 96 sample holes (diameter 3.1 mm) were punched into the gel plate and 91 samples of $5 \mu\text{l}$ were applied together with 5 standards consisting of 3 different amounts of CI-2 protein as well as 2 samples prepared from a Nordal and Hiproly whole milled flour, respectively. The plates were incubated for 2 days and the diameter of the stained precipitates measured.

Values of CI-2 content in F_1 and F_2 seeds were used for statistical analyses. F_2 plants were analyzed for homozygosity or heterozygosity by measuring individually the CI-2 content on 7 seeds from the central spike. F_2 plants in which all 7 seeds gave a low value were considered homozygous *Lys/Lys* and those in which all 7 seeds had a high CI-2 content were classified homozygous *lys/lys*. A value of 6.5 arbitrary CI-2 units gave the best separation between plants that were high and those that were low in CI-2 content. As

heterozygotes were classified those plants which among the 7 seeds gave 1 to 6 seeds containing a CI-2 content above 6.5 arbitrary CI-2 units in agreement with the assumption that a high CI-2 content requires homozygosity for the recessive *lys* gene. The residual seeds from each plant were ground in a cyclone sample mill and used for DBC and KJELDAHL analyses.

2.3. Determination of dye binding capacity (DBC) and Kjeldahl protein

Duplicate samples of barley flour (0.5 and 0.25 g) obtained from the individual F_2 plants in the crosses Nordal x Hiproly and Hiproly x Nordal (bulk F_3 seeds) were analyzed for DBC and KJELDAHL protein as described earlier (7).

2.4. Statistical treatment of results

Quantification of CI-2 in F_2 half seeds derived from reciprocal Hiproly crosses did not produce discrete distributions in all combinations (Figure 1) as is to be expected from the distributions of the involved genotypes (Figure 3). For simplification it was assumed that the individual histograms represent two normal distributions of low and high CI-2 content. The weight of the low content distribution $N(\mu_1, \sigma_1)$ relative to the weight of the high content distribution $N(\mu_2, \sigma_2)$ in the F_2 is expressed by ω . The distributions of the CI-2 content in the F_2 generation of individual crosses is then expressed by

$$f(x) = \omega/\sigma_1 \phi((x-\mu_1)/\sigma_1) + (1-\omega)/\sigma_2 \phi((x-\mu_2)/\sigma_2)$$

The 5 parameters of each distribution (μ_1 , σ_1 , μ_2 , σ_2 , ω) were estimated using FISHERS maximum likelihood method (Table I). The solutions to the equation were solved by a computer program based on the NELDER-MEAD simplex method (16,17). The estimated distributions (exemplified in Figure 2) were tested for goodness of fit by testing the difference between the observed and estimated number in each class using the χ^2 -test. The fitness of the classes using this test were accepted at a 5% level of significance. The large number of observations allowed calculation of the standard deviations of the estimated parameters by the method of HASSELBLAD (4). Parameters estimated in this way can be considered as normally distributed and pairs of parameters

Figure 1. The distribution of inhibitor CI-2 content in F_2 -seeds from reciprocal crosses between Hiproly and the normal lysine genotypes Nordal, Welam as well as CI 4362 (Hiproly sister line).

within reciprocal crosses were consequently tested by the u-test (Table II).

3. RESULTS

3.1. Analysis of CI-2 content in F_1 seeds

F_1 seeds from crosses between Hiproly and Nordal barley were analyzed for their CI-2 content. It can be seen from Table III that the triploid endosperm of the F_1 seeds derived from the crosses Nordal x Nordal and Nordal x Hiproly

Table I**Analysis of distributions of CI-2 content in F_2 seeds obtained from reciprocal Hiproly crosses.**

Crosses	Genera- tion	n	mean of low content CI-2 segregants		mean of high content CI-2 segregants		weight of distributions
			μ_1	σ_1	μ_2	σ_2	
Hiproly x Nordal	F_2	357	6.1	0.7	11.3	1.6	0.80
Nordal x Hiproly	F_2	297	5.0	0.8	8.1	2.0	0.79
Hiproly x Welam	F_2	364	6.5	0.9	12.5	1.8	0.87
Welam x Hiproly	F_2	349	5.8	0.7	9.9	1.4	0.76
Hiproly x CI 4362	F_2	362	8.0	0.8	14.2	1.3	0.85
CI 4362 x Hiproly	F_2	359	7.8	0.9	13.8	1.3	0.83

Table II**A u-test comparison of means, variances and weights of reciprocal Hiproly crosses in the F_2 seed generation.**

Crosses	mean of low content CI-2 segregants	standard error	mean of high content CI-2 segregants	standard error	weight
	μ_1	σ_1	μ_2	σ_2	ω
Hiproly x Nordal	+++	n.s.	+++	n.s.	n.s.
Nordal x Hiproly					
Hiproly x Welam	+++	+++	+++	n.s.	+++
Welam x Hiproly					
Hiproly x CI 4362	++	n.s.	n.s.	n.s.	n.s.
CI 4362 x Hiproly					

+++ : Significance at the 0.01% level. ++ : Significance at the 1% level. n.s. : Not significantly different parameters.

Figure 2. The CI-2 distributions obtained in F_2 (Figure 1) were resolved into two normal distributions by iterative computation using the maximum likelihood function. The resulting parameters are given in Table I.

do not differ significantly from one another. However, the seeds derived from the cross Hiproly x Nordal are intermediate in CI-2 content and are significantly different from the Nordal x Nordal and Nordal x Hiproly seeds as well as the manually selfed Hiproly seeds (Hiproly x Hiproly).

3.2. Analysis of CI-2 content in F_2 seeds

The distributions of high and low CI-2 content in F_2 segregants were different in the reciprocal crosses between Hiproly and the two normal lysine varieties Nordal and Welam (Figure 1). In the reciprocal crosses between Hiproly and CI 4362 (Hiproly isogenic sisterline) two almost identical histograms were obtained (Table I and II). The large variation observed among the F_2

segregants with a high CI-2 content is characteristic of the Hiproly parent line used for the crosses (Figure 3). Analysis of Nordal, Welam and CI 4362 (Figure 3) gave narrow distributions, the content of CI-2 being significantly higher in CI 4362 than in Welam and Nordal, a difference also observably if the low CI-2 content segregants of the crosses involving CI 4362 are compared with those involving Welam and Nordal (cf. Figure 1). It can be seen from the F_2 distributions in Figure 1 that only very few segregants with a low CI-2 content fall into classes characteristic for Nordal, Welam and CI 4362, respectively. This may indicate that the *lys* allele is not entirely recessive.

The 6 individual crosses were tested for a 3:1 segregation of low to high CI-2 content under the simplified assumptions described under 2.4 (Table IV). In the two crosses, Nordal x Hiproly and Welam x Hiproly, segregation did not differ significantly from the assumption of a 3:1 ratio. The other 4 crosses displayed significantly lower numbers of segregants high in CI-2 content than expected. This deficit of high CI-2 segregants was especially pronounced in the crosses Hiproly x CI 4362 and CI 4362 x Hiproly, where the distributions were closely similar in the reciprocal combinations. It should be mentioned that the present analyses do not permit to decide if the differences between the CI-2 content of Nordal/Welam, CI 4362 and Hiproly are due to 3 different alleles of a single gene or due to alleles in 2 different genes. In the latter case the segregation test is not valid.

3.3. Comparison of two screening methods, CI-2 quantification and DBC, for the Hiproly character

Comparison of the two methods, i.e. determination of CI-2 content and DBC, was performed on F_2 plants from the crosses Hiproly x Nordal and Nordal x Hiproly which by progeny analysis had been classified into plants homozygous for high CI-2 content, plants homozygous for low CI-2 content and plants heterozygous for the trait (section 2.2.). Seeds from these individual F_2 plants were analysed for KJELDAHL protein and DBC and the results plotted (Figure 4). Values for the 28 plants judged as homozygous for high CI-2 content were distributed closely

Figure 3. The distribution of inhibitor CI-2 content of the genotypes Nordal, Welam, CI 4362 and Hiproly. 46 seeds of each genotype derived from a field material were analyzed for CI-2 content.

around a regression line located significantly above the regression line obtained for the 24 plants judged as homozygous for a low CI-2 content. The 13 heterozygous plants distributed among either of the two regression lines.

4. DISCUSSION

The content of chymotrypsin inhibitor in F_1 seeds from the Nordal x Hiproly cross deviated significantly from the CI-2 content of the seeds derived from the Hiproly x Nordal cross (Table III). Similar reciprocal differences were observed in the F_2 distributions for the crosses with Nordal as well as Welam (Figure 1).

This reciprocal difference in F_1 and F_2 has previously been observed by ULLRICH and ESLICK (21) in Betses x Hiproly crosses. Analysis of the F_2 showed that both the segregants high and low in CI-2 content contained generally more of this protein when Hiproly was used as a maternal parent in crosses with the normal lysine genotypes Nordal and Welam than in the reciprocal crosses. When Hiproly was used as the paternal

Table III

The t-test of F_1 seeds.

	n	mean of CI-2 content	standard error	t-value	
Nordal x Nordal	12	5.4	1.0	H x N vs N x N, N x H	8.7 ⁺⁺⁺
Nordal x Hiproly	16	6.1	1.5	H x N vs H x H	10.7 ⁺⁺⁺
Hiproly x Nordal	18	9.4	1.6		
Hiproly x Hiproly	12	15.6	1.5		

⁺⁺⁺: Significance at the 0.01% level. Nordal x Nordal and Nordal x Hiproly proved not to be significantly different at the 5% level (F- and t-test).

Table IV

Segregation of high and low content of inhibitor CI-2 in F_2 seeds derived from three reciprocal Hiproly crosses.

Crosses	n	expected	observed	u-test
Hiproly x Nordal	357	0.75	0.80	+
Nordal x Hiproly	297	0.75	0.79	n.s.
Hiproly x Welam	364	0.75	0.87	+++
Welam x Hiproly	349	0.75	0.76	n.s.
Hiproly x CI 4362	362	0.75	0.85	+++
CI 4362 x Hiproly	359	0.75	0.83	+++

⁺⁺⁺: Significance at the 0.01% level, +: Significance at the 5% level. n.s.: Not significantly different from the expected ratio.

parent a general reduction in CI-2 content was observed, and the distinction between high and low CI-2 segregants was unclear (Figure 1 and Table I). As pointed out by ULLRICH and ESLICK (21) a cytoplasmically inherited determinant influencing the expression of the *lys* gene is a possible explanation. Comparison of the distributions (Figure 1) with those of the parental genotypes (Figure 3) shows that Hiproly is the major source of the variations. This variation in Hiproly may be connected to difficulties in avoiding crosspollination.

Analysis of the crosses Hiproly x CI 4362 and CI 4362 x Hiproly supports the isogenic relationship between CI 4362 and Hiproly inferred from morphological and amino acid comparisons of the two Ethiopian genotypes (13,14). Almost identical distributions of CI-2 (Figure 1 and Ta-

ble II) were obtained in both combinations. The genotype CI 4362 displays a higher content of CI-2 than normal lysine genotypes, and this is retained in both the high and low CI-2 segregants of the F_2 generation, indicating that an independent gene, homozygous both in Hiproly and CI 4362, is responsible for this elevated level of CI-2. The resolution achieved with the CI-2 assay will permit a definitive analysis in additional crosses and progeny analyses.

Figure 4. Analysis of F_2 plants (bulked F_3 seeds) derived from the reciprocal crosses between Hiproly and Nordal barley.

Seven individual F_3 seeds from each plant were analyzed for CI-2 content to determine if the plant segregated or was homozygous. The residual grains were ground and the flour was analyzed for DBC and KJELDAHL protein.

- Plants homozygous for high CI-2 content.
- Plants homozygous for low CI-2 content.
- ▲ Plants heterozygous for CI-2 content.
- Hiproly barley.
- Nordal barley.

Despite a good separation of segregants derived from the crosses Hiproly x Nordal and Hiproly x Welam (Figure 1), significant deviations from a 3:1 segregation of low to high CI-2 content were obtained. The crosses Nordal x Hiproly and Welam x Hiproly gave unclear separation of segregants but the estimated segregation ratios were not significantly different from 3:1. The low number of segregants with high CI-2 content obtained in the crosses Hiproly x Nordal and Hiproly x Welam may be related to the difficulties encountered in obtaining progeny from combinations using Hiproly as mother (15). Sterility in such crosses was reported by KARLSSON (9) who separated the sterility gene from the *lys* gene by backcrossing. It is evident from these authors (9,15) that the result of crosses with Hiproly is highly dependent on the environmental conditions at the time of fertilization. The present data points towards a monogenic recessive inheritance of the high CI-2 content in crosses with Hiproly. Monogenic recessive inheritance for the high CI-2 content was displayed by the 13 heterozygous plants identified among the Hiproly x Nordal and Nordal x Hiproly F_2 plants (Table V). These 13 heterozygotes were found among 28 plants with high and 24 plants with a low CI-2 content. Unfortunately a random sample of F_2 plants was not analyzed and it remains therefore open to what extent the low number of heterozygotes is due to the sampling method applied.

Genotypic classification of 65 F_2 plants in the crosses between Hiproly and Nordal allowed comparison between the immunological CI-2 quantification method and the DBC method which is based on bulked F_3 seeds from the F_2 plants. Results presented in Figure 4 show that the plants identified as homozygous for high and

low CI-2 content, respectively, were classified in the same category by the DBC method. On the other hand, plants segregating for the CI-2 content could be classified only by determination of CI-2 content of individual seeds. The good agreement between the DBC determinations and the CI-2 quantification method reveals that a high CI-2 content is diagnostic for the presence of the *lys* gene and that the CI-2 quantification based on single seeds is an efficient method in breeding for high lysine content conditioned by the *lys* gene.

ACKNOWLEDGEMENT

I am grateful to Fil. Dr. L. MUNCK, Lic. Agro. H. DOLL, Fil. Dr. A. TALLBERG, cand. scient. S. AASTRUP and J. MUNDY for helpful discussions, and to cand. stat. L. SEJERSEN and S. HORNSGÅRD for the statistical work. The skilled technical assistance of Ms. B. STILLING is also gratefully acknowledged and I am indebted to the personal of the Phytotron in Stockholm for growing the plants.

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Table V

Segregation of CI-2 content in seeds of F_2 plants.

Cross	Mean of seeds		Mean of seeds		Expected ratio	Observed ratio
	<8.5 CI-2 units	standard error	>8.5 CI-2 units	standard error		
Hiproly x Nordal	5.6	1.1	10.1	1.0	3:1	36:13 n.s.
Nordal x Hiproly	5.6	0.8	10.6	1.4	3:1	31:9 n.s.

n.s. = no significant difference from 3:1.

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