Phytochemistry, Vol 24, No. 8, pp. 1717–1723, 1985.
Printed in Great Britain.

0031–9422/85 $3.00+0.00

VARIATION OF THE LEGUMIN SEED STORAGE PROTEIN AMONGST *VICIA* SPECIES

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(*Revised received* 12 *November* 1984)

Key Word Index—*Vicia*; Leguminosae; storage protein; legumin.

Abstract—Variation in legumin, the major seed storage protein of *Vicia faba* was investigated in different (primitive and cultivated) varieties of *V. faba* and in other primitive *Vicia* species. Qualitative variation in legumin subunit patterns on gel electrophoresis was less within the species *V. faba* than the variation between *Vicia* species. However, the large seeded modern *V. faba* cultivars showed much increased levels of the 'main' legumin subunit pairs. Analyses of amino acid composition, and nitrogen and sulphur content did not show systematic variation between the samples tested, and suggested that breeding and selection had not decreased protein content or nutritional quality. It was concluded that the heterogeneities of legumin genes in the *Vicia* species examined are comparable, and that selection for a large-seeded phenotype in *V. faba* has had the effect of increasing the expression of a subset of legumin genes, those encoding the 'main' subunit pairs.

INTRODUCTION

Legumin, the major storage protein in seeds of *Vicia faba*, is a hexameric molecule (M_r approx. 380000) composed of six subunit pairs held together by non-covalent forces; each subunit pair in turn contains an α(acidic) and a β (basic) subunit joined by one or more disulphide bonds [1, 2]. Thus on treatment with SDS the molecule dissociates into subunit pairs, which will further dissociate to individual α and β subunits if treated with reducing agents such as 2-mercaptoethanol. The two-stage dissociation of legumin can be exploited to identify legumin subunits in a complex mixture of proteins such as a total protein extract of seeds. A two-dimensional SDS-polyacrylamide gel electrophoretic analysis technique is most conveniently used, running the proteins non-reduced in the first dimension and reduced in the second, so that disulphide-bonded subunits separate in the second dimension and appear off the 'diagonal' of non-disulphide linked polypeptides. This technique was used to identify ten different legumin subunit pairs in *Vicia faba* cv. Felix [3], in which the α subunits varied considerably in M_r (23000–58000) whereas the β subunits were relatively invariant (M_r 21000–23000). In the present paper structural variation in legumin is assessed in six *Vicia* species and two *Vicia faba* cultivars in order to compare variation between species to that within a species, and to attempt to assess the effect of breeding for a large-seeded phenotype on seed protein genes and their expression.

Variation in *Vicia faba* protein content and amino acid composition has been considered by Lafiandra *et al.* [4] and Sjödin [5] amongst other authors, and qualitative and quantitative variation in storage proteins have been investigated in *Pisum* [6] and *Vicia* [7, 8], but we are not aware of other studies attempting to compare intra- and inter-specific variation of storage proteins in *Vicia*.

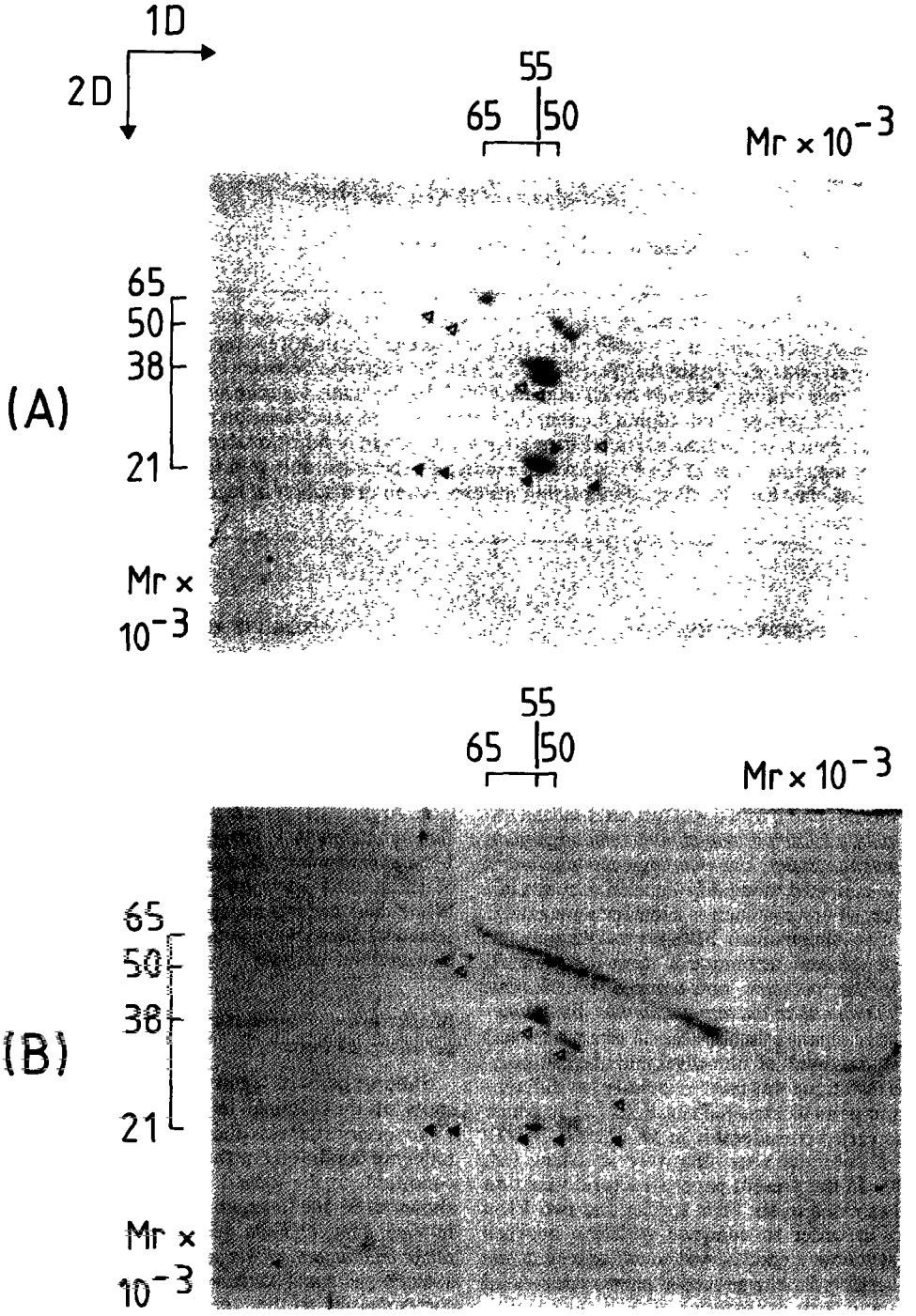
RESULTS AND DISCUSSION

Choice of materials

The following seeds were selected for analysis: *Vicia faba* maj. cv. 'Triple White' (commercial broad bean); *Vicia faba* min. cv. 'Maris Bead' (modern, commercial field bean); *Vicia faba* min. cv. 182 (a primitive *V. faba* cultivar from Afghanistan); *Vicia galilea*; *Vicia narbonnensis* (both closely related to *V. faba*); *Vicia villosa*; *Vicia pannonica*; *Vicia atropurpurea* (all more distantly related to *V. faba*); of these, only *Vicia faba* cv. 'Maris Bead' and 'Triple White' had undergone significant artificial breeding and selection. Seeds were grown under conditions where no nutrient was limiting.

Identification of legumin polypeptides by two-dimensional gel electrophoresis

Subunit pairs of legumin were recognised as pairs of spots 'off the diagonal' in gels of total protein extracts of *Vicia* species after two dimensional SDS-PAGE with non-reducing conditions in the first dimension and reducing conditions in the second (Fig. 1). These spots have been shown to be due to legumin or legumin-like polypeptides in *Vicia faba* cv. 'Felix' [3] by immunoprecipitation and other techniques. In the present paper we divide legumin subunit pairs into three types based on their M_r values, corresponding to the types observed in cv. 'Felix'; 'large' (60000–80000 M_r) 'main' (55000–58000 M_r) and 'small' (30000–50000 M_r). *Vicia faba* cv. 'Maris Bead' displays a similar pattern of legumin polypeptides to that of *Vicia faba* cv. 'Felix', with the polypeptides of the main subunit pairs of legumin (M_r ca 56000) present in very much larger amount than any other subunit pair. Small amounts of legumin subunit pairs of higher M_r are present (M_r


1D
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(A)
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(B)
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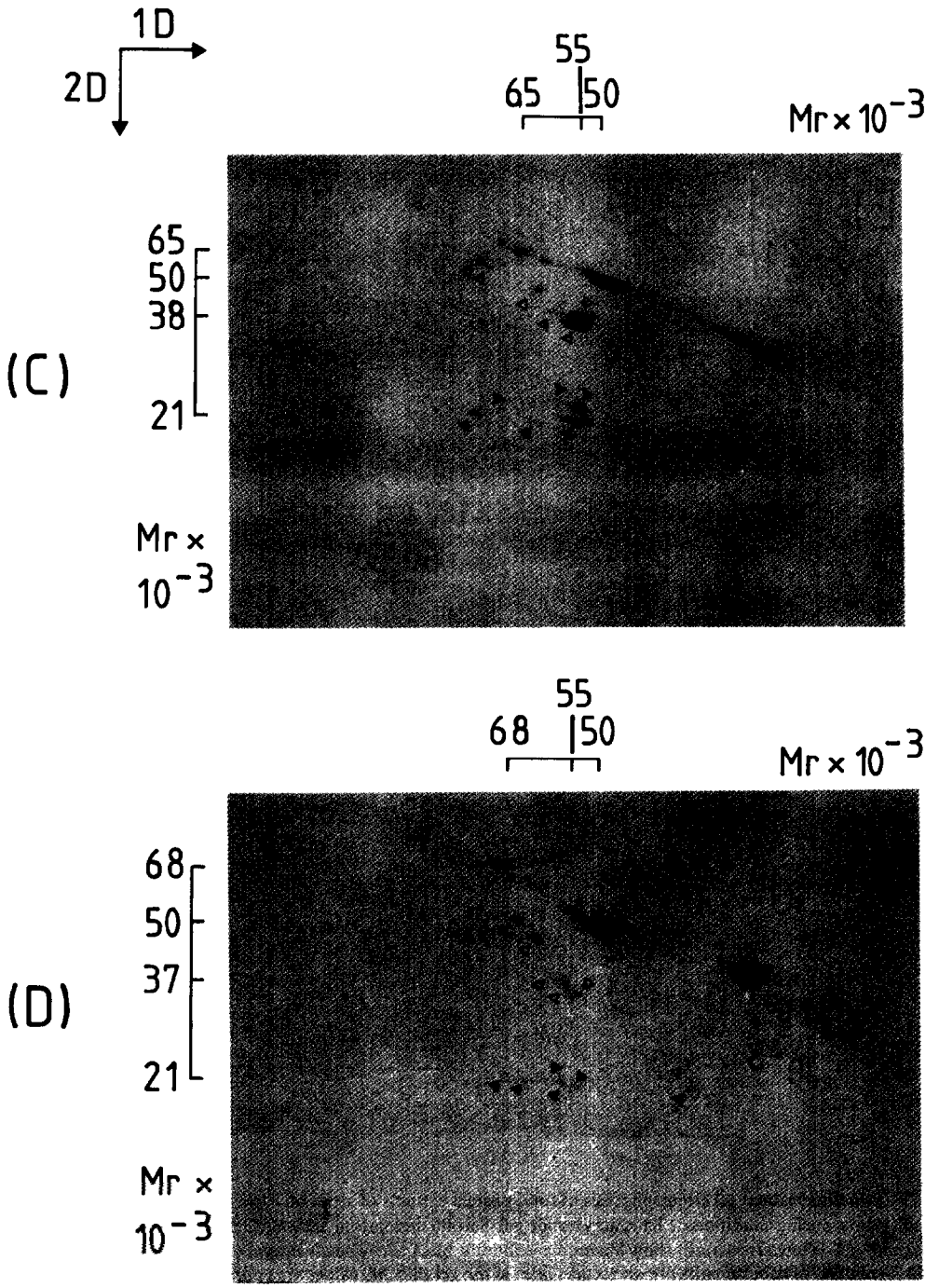
21
Mr x
10-3

Fig. 1. A, B


1D
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55
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(C)
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68
50
37
21
(D)
Mr ×
10⁻³

Fig. 1. C, D

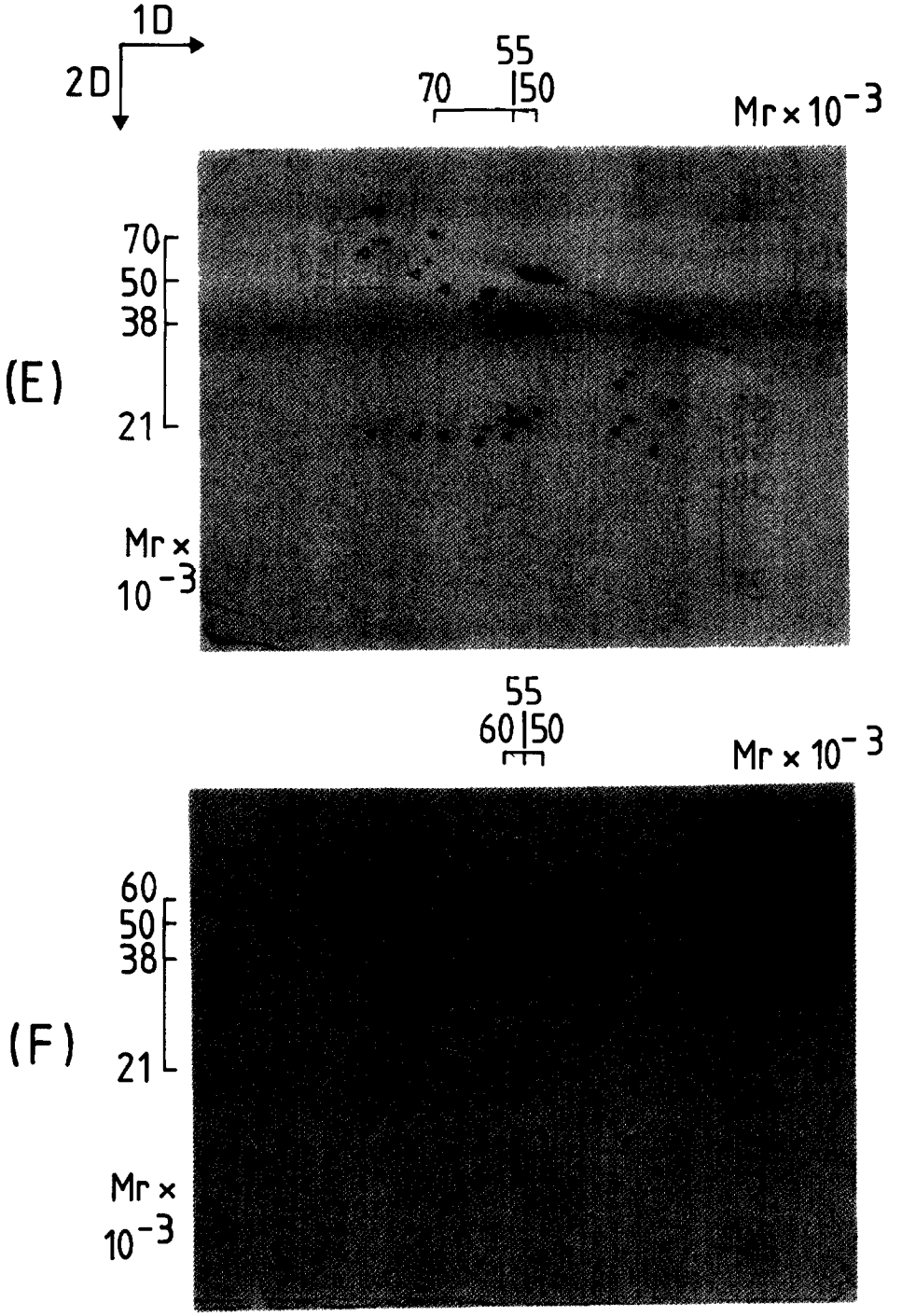

Fig. 1. Two dimensional gel electrophoresis of total protein extracts of seeds of *Vicia* spp. First dimension; SDS-PAGE, non-reducing conditions, 12.5% acrylamide gel. Second dimension; SDS-PAGE, reducing conditions, 17% acrylamide gel. White arrowheads show legumin α-subunits, black arrowheads legumin β-subunits. Corresponding α-and β-subunits of subunit pairs are on vertical lines in the gel slab. M_r values are approximate. Some arrowheads indicate more than one subunit. (A) *Vicia faba* cv. 'Maris Bead'. Note high legumin content, predominantly of 'main' legumin subunit pairs. (B) *Vicia faba* cv. 182 (primitive variety). Legumin content much lower than (A), but 'main' legumin subunit pairs predominant. (C) *Vicia narbonnensis*. Note broad similarity to (A), but with increased proportion of 'big' legumin subunit pairs. (D) *Vicia galilea*. Note low legumin content, 'main' legumin subunit pairs much reduced. (E) *Vicia villosa*. Note equal amounts of most legumin subunit pairs, more complex pattern with some legumin subunit pairs containing very large α-subunits. (F) *Vicia pannonica*. Broadly similar to (E), but size distribution of legumin subunit pairs less extensive.

65 000–70 000) as are small amounts of a low M_r subunit pair (M_r *ca* 40 000). A total of five subunit pairs could be identified, compared to the nine identified for cv 'Felix'; however, the two cultivars are similar in the preponderance of the main legumin subunit pairs over all others. The primitive *V. faba* cv 182 showed a qualitatively similar pattern, but contained much less legumin relative to overall protein. Only one high M_r subunit pair could be identified, but more low M_r subunit pairs were present, giving a total of six subunit pairs. The main subunit pairs were at M_r 57 000; although not as strongly stained as 'Maris Bead' they were present at higher amount than other subunit pairs. A further *V. faba* cv. 'Triple White', a large seeded broad bean, gave a broadly similar pattern, with 5–6 subunit pairs identifiable, and a predominant subunit pair at *ca* 56 000 M_r (results not shown).

The two species closely related to *V. faba* gave results differing from each other. Whereas the *V. narbonnensis* legumin subunit pattern was very similar to *V. faba*, with predominant subunit pairs at *ca* 60 000 M_r, and minor subunit pairs at higher and lower M_r, the *V. galilea* pattern showed a low legumin content, with no predominant subunit pairs. A low M_r subunit pair was observed, at *ca* 35 000 M_r, and other subunit pairs in the region 50 000–70 000 M_r. Of the remaining three species, *V. villosa* gave a pattern somewhat similar to *V. faba*, with high M_r, low M_r, and main legumin subunit pairs, although the main legumin subunit pairs were not present in large excess over the others. This species had a subunit pair of very high M_r, *ca* 90 000. *V. pannonica* gave a pattern similar to *V. galilea*, in that the legumin level was low, and predominant legumin subunit pairs were not apparent. Again, subunit pairs in the low, high and main M_r ranges were observed. *V. atropurpurea* also gave this type of pattern. In all the subunit pairs observed, the M_r of the smaller (β) subunit was *ca* 20 000, whereas the M_rs of the larger (α) subunit varied. This suggests that the sequence of the β subunit is more conserved, and thus under greater structural constraint, than that of the α subunit in legumin subunit pairs.

Estimation of proportion of legumin in total protein

An estimate of legumin contents of the different *Vicia* samples tested was obtained by densitometric scanning of gels containing total protein extracts of the seed meals. Legumin peaks on the densitometric scans were identified by their disappearance under reducing conditions, and the area of the peak was compared to the total peak area. Although this densitometric technique is inherently inaccurate since the relationship between amount of protein and Coomassie Blue staining is non-linear, these figures show the expected trend in legumin content, with *V. faba* cv. 'Felix' and 'Maris Bead' having legumin contents significantly higher than any other sample (45–50%). *V. faba* cv. 'Triple White', *V. narbonnensis* and *V. villosa* had medium legumin contents (25–30%) whereas the other samples had low legumin contents (15–25%). Legumin levels can be affected by environmental conditions, but effects that would be significant in terms of the present study are only produced by severe sulphur deprivation [9, 10]. The observed trends are thus likely to be the result of genetic rather than environmental factors. On the basis of the 'primitive' varieties, species to species variation in legumin content does not appear to be significant; however, within the species *V. faba*, agricultural selection and breeding has not only increased seed size but has tended to increase legumin content also.

Amino acid and chemical analysis

Nitrogen and sulphur contents, and amino acid analyses were carried out on the seed meals studied in order to show whether significant intra- and inter-species variation could be detected. The effects of environmental factors on the observed amino acid contents and % N and % S levels are not likely to be as significant as genetic factors in the present study since seeds were produced under conditions where nutrient was not limiting [11]. Results of the chemical analyses are given in Table 2. % N varied from 3.9 (*V. narbonnensis*) to 7.2 (*V. faba* cv. 'Triple White'); both the larger seeded cultivated *V. faba* seeds had high % N (> 5.5) whereas the primitive *V. faba* had lower % N (4.9). Variation within *V. faba* was comparable to variation between species and no clear pattern of variation was observed. A similar pattern was shown by sulphur levels; variation occurred over the range 0.19–0.31%, and levels in the large seeded *V. faba* were comparable to others. These data suggested that breeding for large seeds had not decreased N or S content; in fact, if anything, higher N content has been successfully bred for. N content did not correlate with legumin content, but S content showed a weak correlation with legumin content ($R = 0.70$). Amino acid compositions of the different

Table 1. Analysis of legumin of *Vicia* species

Seed	No. of subunit pairs	Predominant subunit pairs at approx 56 000 M_r	Approx. legumin content % of total protein
V. faba cv 'Felix'*	9	+	45
V. faba cv. 'Maris Bead'	5	+	48
V. faba cv. 'Triple White'	5–6	+	30
V. faba cv. 182	7–9	+	22
V. narbonnensis	8–9	+	28
V. galilea	6	–	18
V villosa	9	–	29
V. pannonica	6	–	15
V. atropurpurea	5	–	23

*Data taken from ref. [2].

Table 2. Nitrogen and sulphur analyses of *Vicia* species

Seed	% N	% S
V. faba cv. 'Maris Bead'	5.7	0.29
V. faba cv. 'Triple White'	7.2	0.20
V. faba cv. 182	4.9	0.21
V. narbonnensis	3.9	0.21
V. galilea	4.8	0.19
V. villosa	5.4	0.31
V. pannonica	5.3	0.20
V. atropurpurea	6.1	0.19

Values are means of triplicate determinations; variation beween determinations was $\leqslant 10\%$.

meals are given in Table 3. All the meals analysed showed the typical legume seed amino acid pattern—high levels of acidic amino acids (Asp and Glu) and low levels of the nutritionally limiting sulphur amino acids (Cys and Met). The contents of Met and Cys varied between species; Met was relatively constant in the *V. faba* varieties tested but Cys was higher in the primitive small seeded *V. faba* than the cultivated varieties. Most other *Vicia* species had higher Met and Cys contents than *V. faba*. No clear correlation between amino acid composition and legumin content, or % N, or % S could be drawn, although it was clear that a high legumin content did not result in increased Met and Cys levels in the amino acid composition, nor did it necessarily depress the levels of sulphur amino acids. The amino acid composition of legumin in the different *Vicia* species and cultivars may itself vary, particularly with regard to the sulphur amino acids, as is known to be the case in different pea lines [12]. Multiple factors are clearly involved in determining amino acid composition which are beyond the scope of the present analysis. Chemical analyses of this type are clearly not a useful tool in distinguishing intra- from inter-specific variation in seed proteins unless applied to limited sets of samples produced under carefully controlled conditions.

All the amino acid analyses showed the presence of unidentified peaks, presumably due to non-protein amino acids; these were not characterized.

Conclusions

The data presented lead to the following conclusions:

1. In the *Vicia* samples studied, quantitative variation in amino acid composition of seeds within a species is comparable to that between species. Similarly, variations in N and S content between species and within a species are comparable.
2. Breeding *V. faba* for increased seed size has not decreased N or S content, nor has it appreciably altered the amino acid composition.
3. Variation in legumin content between wild-type *Vicia* species is less than variation between cultivated and primitive varieties of *V. faba*; the cultivated varieties considered tend to have higher legumin contents, although this may not be true for all cultivars.
4. The numbers of subunit pairs in legumin and their heterogeneity in size are comparable over the range of samples tested. Increase in legumin content, or seed size does not lead to an increase in the number of legumin subunit pairs; instead, a subset of legumin subunits are increased in amount. This suggests that the number and types of genes encoding legumin are comparable in all

Table 3. Amino acid composition (g/16 g N) of seed meals

	V. faba							
	cv. 'Maris Bead'	cv. 'Triple White'	cv. 182	*V. narbonnensis*	*V. galilea*	*V. villosa*	*V. pannonica*	*V. atropurpurea*
Asp	8.52	8.65	7.33	10.27	10.42	8.61	10.36	10.05
Thr	2.67	2.57	2.56	3.95	3.00	3.00	3.69	3.22
Ser	3.74	3.79	3.35	4.82	4.40	3.46	4.77	4.09
Glu	17.48	15.95	12.66	19.42	18.19	14.00	18.13	18.41
Pro	2.40	3.00	2.77	3.82	4.03	2.29	3.87	3.25
Gly	3.51	3.50	2.95	3.83	3.35	2.90	4.23	3.66
Ala	4.31	4.29	3.80	5.26	4.17	4.08	5.22	4.77
Val	3.51	3.50	3.13	4.78	4.34	3.78	4.72	4.07
Met*	0.50	0.46	0.47	0.68	0.50	0.61	0.46	0.64
Ileu	2.99	3.05	2.70	3.92	3.79	3.04	4.23	3.36
Leu	5.96	6.42	5.07	6.97	7.08	5.44	7.47	6 56
Tyr	2.59	2.64	2.33	2.98	2.57	2.62	3.24	3.07
Phe	3.24	3.32	2.94	4.30	3.99	3.31	4.44	4.01
His	1.69	1.72	1.54	2.22	1.80	1.76	2.57	2.08
Lys	4.80	4.80	5.00	6.71	6.26	4.94	6.41	5.82
Arg	5.39	4.65	5.70	7.84	7.45	4.96	6.11	5.04
Cys*	1.00	0 98	1.30	1.75	1.55	1.59	1 07	2.05
Total	68.32	73.29	65.62	92.51	86.87	70.39	90.98	83.93

*Determined after performic acid oxidation Values are taken from duplicate 24 hr hydrolysis; variation between hydrolyses was $\leqslant 10\%$.

these species, but expression of a subset of legumin genes can be favoured; cultivation in *V. faba* has increased the expression of these genes encoding the 'main' legumin subunit pairs. This process could occur by gene amplification or by control of mRNA production. Studies of the genomes of *Vicia* species and cultivars to determine the numbers and types of legumin genes present will be required to clarify the course of this inter- and intra-specific variation.

EXPERIMENTAL

Seeds of *Vicia* species were obtained from Tyneside Seed Stores, Gateshead, Tyne & Wear, U.K., or were supplied by Dr. P. Gates, Department of Botany, Durham University SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to ref. [13], and as modified in ref. [3] for two dimensional gels where the first dimension is run under non-reducing conditions and the second dimension is run under reducing conditions. Acrylamide concns of 12.5% and 17% respectively were used for first and second dimension gels. Densitometric scanning of strips cut from stained and destained gels was carried out on a Gilford model 2000 densitometer; peak areas were estimated by cutting out the appropriate areas of the recorder trace and weighing.

Nitrogen analyses were carried out by a micro-Kjeldahl technique, as described in ref. [14]. Sulphur was estimated as sulphate [15]. Amino acid analysis was carried out on a Locarte Autoanalyser by conventional methods [16, 17]; methionine and cysteine were estimated as methionine sulphoxide and cysteine acid after performic acid oxidation [18].

Acknowledgements—We thank Dr P Gates for supplying seeds, and Mr. R. Swinhoe for technical assistance.

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