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# Isozyme gene markers in *Vicia faba* L.

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**Summary.** This study was conducted to assess the genetic basis of the variability observed for the glutamate oxaloacetate transaminase (GOT), superoxide dismutase (SOD), esterase (EST), and malate dehydrogenase (MDH) isozyme systems in different open-pollinated *Vicia faba* varieties. Individual plants showing contrasting zymogram patterns were simultaneously selfed and cross-combined. Crossing was unsuccessful in producing progeny, and only selfed progenies were suitable for genetical analysis of isozyme variability. Three zones of GOT activity were made visible. The isozyme of GOT-2 and GOT-3 zones were dimeric and under the control of three alleles at the *Got-2* locus and two alleles at the *Got-3* locus, respectively. The isozymes of the GOT-1 zone did not show any variability. Three zones of SOD isozyme activity were made visible. The isozymes occurring in the SOD-1 (chloroplastic isozyme form) and SOD-2 (cytosol isozyme form) zones were dimeric and under the control of two alleles at the *Sod-1* and *Sod-2* loci. The isozyme visualized in the SOD-3 zone (mitochondrial isozyme form) were tetrameric and under the control of two alleles at the *Sod-3* locus. Apparently the isozymes made visible in the most anodal esterase zones EST-1, EST-2, and EST-3 were monomeric, and the occurrence of two alleles at each of two different loci explained the variability observed in the EST-2 and EST-3 zones. For MDH, only two five-banded zymogram pattern types were found, and every selfed progeny showed only one of the two zymogram type, indicating that each individual possessed fixed alleles at the loci controlling MDH isozyme. *Got-2, Got-3, Sod-1, Sod-2,* and *Sod-3* appear to be five new isozyme gene markers that can be useful in *Vicia faba* breeding for linkage study, varietal fingerprinting, outcrossing rate estimate, and indirect selection for quantitative characters.

**Key words:** *Vicia faba* – Faba bean – Isozymes – Marker genes

## Introduction

In the "Third conspectus of genetic variation within *Vicia faba*", Ward and Chapman (1986) reviewed the status of genetic research in faba bean and reported that 129 alleles at 27 nuclear loci had been identified in that species (Table 1). This indicates that an average of five alleles at four to five loci can be used as genetic markers for each *Vicia faba* ($2n=12$) chromosome. This is an extremely small number of gene markers for such important crop species in the Western Europe and Mediterranean areas.

**Table 1.** Symbols and number of alleles for known morphological gene markers in *Vicia faba* L.

| Feature (organ or cell) | *Locus* (no. of alleles) |
|---|---|
| Leaf | *un* (7) |
| Stem and inflorescence | *ti* (5)[1], *dw* (5), *rs* (2), *lfp* (2), and *efd* (1) |
| Flower and stipule | *ww* (1), *sp* (6)[1], *cf* (1), and *dp* (30) |
| Pollen shape and fertility | *po* (3), *ms* (3), *cms* (4), and *Rf* (4) |
| Seed | *Sc* (16), *V* (6), *y* (5), *r* (2), *sn* (1), *it* (1), *Hi* (4), and *n* (1) |
| Whole plant | *vs* (1) |
| Interaction with bacteria, fungi, and virus | *sym* (1), *Af* (6), *Fr* (9), and *bym* (2) |

[1] Alleles at this locus control character expression in both the indicated organs

The exact chromosomal location of these genes, however, is not known although Sjödin (1971) gave some indications about the linkage map for the genes controlling flower and seed coat color.

The 27 nuclear loci identified control the expression of one or more morphological trait and none encode enzymatic or nonenzymatic protein. However, many reports deal with *V. faba* enzymes and isozymes (Hill-Cottingham 1983), but the genetic basis for the inheritance of certain isozymes has been rarely investigated (Ward and Chapman 1986).

Many applications in plant breeding require the use of one or a few isozyme markers whose genetic control or map position in the genome need not be known (Tanksley 1983). In *V. faba,* isozyme markers have been used for studying taxonomical relationships with other *Vicia* species (Yamamoto et al. 1982), identification of cultivars, inbred lines and hybrids (Bassiri and Rouhani 1977; Gates and Boulter 1979, 1980), and estimation of outcrossing rate (Peat and Adham 1984).

The availability of information on the genetic control of isozymes may increase the usefulness of the isozymes in *Vicia faba* breeding, expecially in linkage study, outcrossing rate estimate based on multiloci parameters, and indirect selection for quantitative trait loci.

This paper presents evidence on the genetic control and subunit composition of three isozyme systems: glutamate oxaloacetate transaminase (GOT; E.C.2.6.1.1.), superoxide dismutase (SOD; E.C.1.15.1.1.), and esterase (EST; E.C.3.2.1.), and the zymogram pattern of malate dehydrogenase (MDH; E.C.1.1.1.37.).

## Materials and methods

### *Plant material*

Several $S_0$ individuals or parental plants were electrophoretically surveyed in April 1986. The $S_0$ individuals were identified in the following open-pollinated varieties or populations: Alto (France), Var. 312 (Greece), Gemini (Italy), VT-1, 83-C, LA1, LA2, LA4, LA11, LA16, LA40, LA28BA, LA63, and PAM (Italy). Alto and Var. 312 were small-seeded varieties (minor type: 100 seed weight below 90 g); Gemini showed medium seed size (equina type: 100 seed weight about 120 g); VT-1, PAM, and all the LA populations were large-seeded varieties (major type: 100 seed weight above 140 g). These varieties were grown in the field of the Experimental Station of the University of Tuscia at Viterbo, Italy. Plants that showed single-, double-, triple-, or quintuple-banded phenotypes in the identified zones of activity in the zymogram pattern for GOT, SOD, EST, and MDH were simultaneously crossed with each other and selfed.

The majority of the cross combinations did not set seeds or produced only one or two $F_1$ seeds. This unsuccessful cross-hybridization was probably due to the fact that the parental plants of a cross-combination very often belonged to different botanical groups (i.e., minor × major combination, etc.); in this situation a sort of unilateral incompatibility may be expressed (Abdalla 1977) leading to very poor $F_1$-seeds set. Therefore, only seeds obtained upon self-fertilization were used in this study.

The resulting progenies were planted in pots filled with soil, sand, and peat moss (1:1:1 ratio) and grown in the greenhouse in the fall of 1986.

### *Isozyme analysis*

*Extraction.* Crude extracts were obtained from the three youngest and unexpanded leaves of 3–4 week-old plants using Carlson's buffer (0.1 *M* Tris-HCl, 0.1 *M* KCl, 0.005 *M* EDTA, 0.04 *M* 2-Mercaptoethanol, 0.1 *M* sucrose, pH 7.5) as modified by Hart (1982) at a tissue : buffer ratio of 1 mg : 4 µl. Extracts were centrifuged at 6,000 rpm, 4 °C for 7 min. The supernatant was either used directly for electrophoresis or stored at −80 °C.

*Electrophoresis.* The composition of the gels and buffers used for resolving GOT, SOD, and EST isozymes in polyacrylamide gels is reported in Table 2. Forty µl of supernatant of each crude sample extract was used for gel analysis. Electrophoresis was performed in a Protean I (Biorad, Richmond/CA) vertical apparatus containing two gels. The running conditions were as described in Montebove et al. (1987) and De Pace et al. (1988).

MDH isozymes were electrophoresed in starch gel containing 0.5 m*M* L-Histidine-monohydrochloride and 12% starch (Connaught) adjusted to pH 7 with 1*N* NaOH; tray buffer was 0.15 *M* Tris adjusted to pH 7 with citric acid. After boiling and evacuation to remove air bubbles, the gel was poured into a plexiglass frame 20 × 15 cm and cooled before use. After the gel hardened, it was transferred on a cooling plate kept at 4 °C and placed on a horizontal apparatus.

Whatmann 3MM filter paper wicks 6 × 12 mm were used to absorb 18 µl of centrifuged sample extract and were inserted in a narrow slit made with a thin spatula, 1.5 cm from an edge. Fifteen to 20 samples were run on each gel. Electrophoresis was started at 150 V and after 20 min, wicks were removed and gels continued to run for 6 hours. After electrophoresis, gels were horizontally cut in three slices, each 3 mm thick.

*Staining.* GOT, SOD, and EST were made visible as described by De Pace et al. (1988). MDH isozymes were made visible by submerging the middle starch gel slice in a 50 ml staining solution prepared as follow: 13 mg NAD, 10 mg NBT, 3 mg PMS, 10 ml of 0.3 *M* malic acid pH 7.5, 20 ml 0.2 *M* Tris-HCl pH 8.0, and 20 ml distilled water. After 30 min at 37 °C, blue bands appeared in the gel slice at the position of MDH isozyme activity.

## Results

### *Glutamate oxaloacetate transaminase, GOT*

Three zones of isozyme activity were observed for GOT. The three zones were designated in sequential order as GOT-1 (the anodal zone), GOT-2, and GOT-3 (the cathodal zone) (Fig. 1).

The isozymes in the GOT-1 zone were made visible in one monomorphic band in all the surveyed plants.

Five bands of isozyme activity were observed in the GOT-2 zone among the analyzed plants. The isozymes made visible in band 1 co-migrated to the same position as the isozymes of the GOT-1 zone. In some plants, band 1 was made visible alone (phenotype $F^FF^F$ in Fig. 1 B), but in others it was found together with bands 2 and 3 (phenotype $F^FF$ in Fig. 1 A–C) or with bands 3 and 5

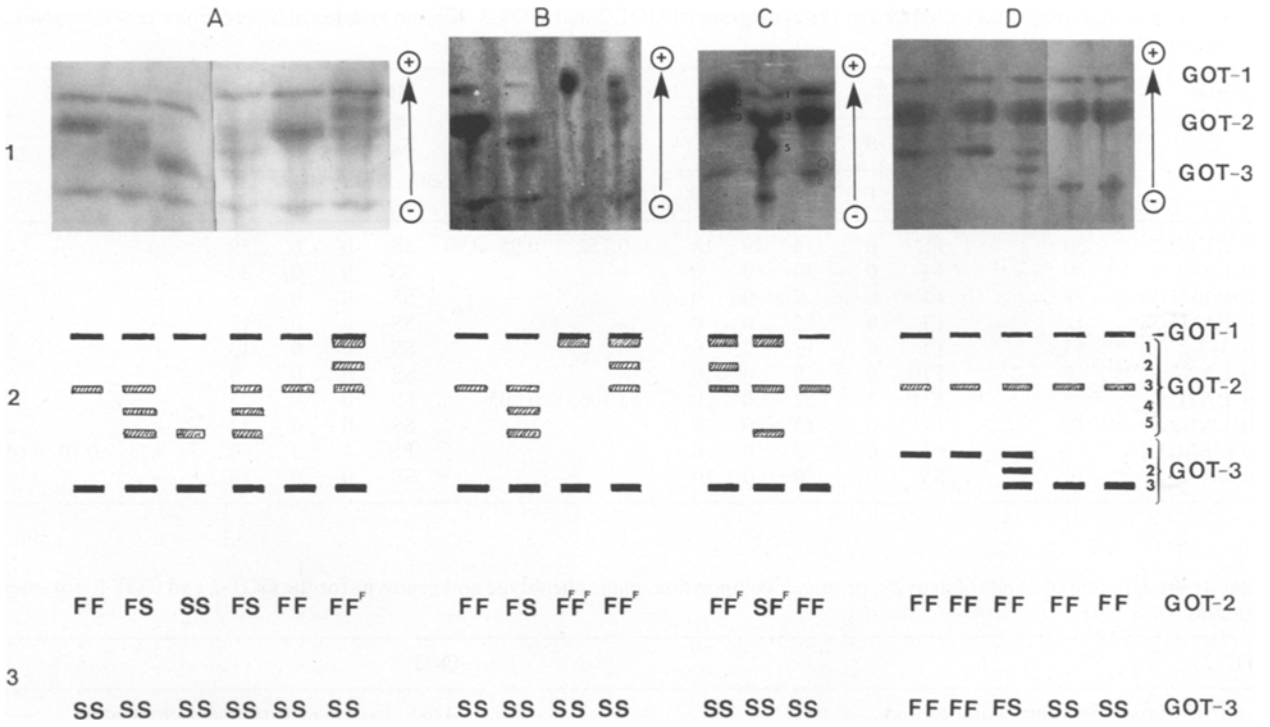


**Fig. 1 A–D.** Zymogram *1*, drawing of the bands of isozyme activity *2*, and phenotype code *3*, for GOT from leaf extract of different groups of plants **A–D** of *Vicia faba*. The numbers in the zymogram pattern **C** indicate the bands analogous to those reported in the band drawing section. *Arrow* denotes the direction of migration in the gel; + and − denote anode and cathode, respectively

**Table 2.** Composition of the stacking gels, resolving gels, and tray buffers used for isozyme electrophoresis in polyacrylamide gels (amount for one 16 × 14 × 0.15 cm gel)

| Isozyme system | Composition of resolving gel | | Composition of stacking gel | | Tray buffer |
|---|---|---|---|---|---|
| | Stock solution | Amount | Stock solution | Amount | |
| GOT | – 1 *M* Tris-HCl (pH 8.9) | 4.2 ml | – 0.416 *M* Tris-HCl (pH 6.9) | 0.6 ml | 0.04 *M* Tris-glycine (pH 8.3) |
| | – 28.0% acrylamide + 0.735% Bis-acrylamide | 8.4 ml | – 10% acrylamide + 2.5% Bis-acrylamide | 1.2 ml | |
| | – 0.114% ammonium persulfate | 21.0 ml | – 40% Sucrose | 2.4 ml | |
| | – TEMED | 10 µl | – 0.5% ammonium persulfate | 0.6 ml | |
| | | | – TEMED | 10 µl | |
| EST SOD | – 0.8 *M* Tris-citrate (pH 9.0) | 10.56 ml | – 0.8 *M* Tris-borate (pH 8.5) | 0.31 ml | 0.2 *M* Tris-borate (pH 8.5) |
| | – 0.15 *M* $Na_2$-EDTA | 0.34 ml | – 0.15 *M* $Na_2$-EDTA | 0.05 ml | |
| | – 20.41% acrylamide + 0.54% Bis-acrylamide | 16.46 ml | – 6.66% acrylamide + 0.37% Bis-acrylamide | 3.60 ml | |
| | – 0.56% ammonium persulfate | 6.24 ml | – 0.56% ammonium persulfate | 0.84 ml | |
| | – TEMED | 10 µl | – TEMED | 7.5 µl | |

(phenotype $F^FS$ in Fig. 1 C). The isozymes of band 2 were always made visible together with those in bands 1 and 3 (phenotypes $F^FF$ in Fig. 1 A–C). In some plants the isozymes of band 3 were made visible alone (phenotype FF in Fig. 1 A–D), but in other plants they were found in combination with those of the other bands (phenotypes FS and $F^FF$ in Fig. 1 A–C; phenotypes $F^FS$ in Fig. 1 C). The isozymes of band 4 were always made visible together with those in bands 3 and 5. The isozymes of band 5 were made visible alone (phenotype SS in Fig. 1 A) or simultaneously with bands 1 and 5 (phenotype $F^FS$ in Fig. 1 C).

All bands observed in the GOT-2 zone showed a staining intensity which was approximately three-fold that observed for the bands in the GOT-1 and GOT-3 zones.

**Table 3.** Phenotypic frequencies observed in the zymogram of GOT-2 and GOT-3 isozyme systems in $S_1$ progenies of *Vicia faba* L. $S_0$ parental plants

| Parental plant ($S_0$) | No. plants in $S_1$ progeny | GOT-2 | | | | | $\chi^2$ | $P$ | GOT-3 | | | | $\chi^2$ | $P$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $S_0$ | $S_1$ | | | | | | $S_0$ | $S_1$ | | | | |
| | | | $F^FF$ | FF | FS | SS | | | | FF | FS | SS | | |
| 1) ALTO-2 | 59 | FS | 0 | 16 | 29 | 14 | 0.152 | 0.95–0.90 | SS | 0 | 0 | 59 | – | |
| 2) 83C-1 | 30 | FF | 0 | 30 | 0 | 0 | – | | SS | 0 | 0 | 30 | – | |
| 3) PAM1-1a | 9 | FF | 0 | 9 | 0 | 0 | – | | SS | 0 | 0 | 9 | – | |
| 4) PAM1-3a | 12 | FF | 0 | 12 | 0 | 0 | – | | SS | 0 | 0 | 12 | – | |
| 5) 312-2 | 13 | FF | 0 | 13 | 0 | 0 | – | | SS | 0 | 0 | 13 | – | |
| 6) LA2-2 | 9 | FF | 0 | 9 | 0 | 0 | – | | SS | 0 | 0 | 9 | – | |
| 7) LA11-1 | 25 | $F^FF$ | 3 | 22 | 0 | 0 | 53.100 | < 0.005 | SS | 0 | 0 | 25 | – | |
| 8) LA16-1 | 13 | FF | 0 | 13 | 0 | 0 | – | | SS | 0 | 0 | 13 | – | |
| 9) GEMINI-1 | 7 | FF | 0 | 7 | 0 | 0 | – | | FS | 4 | 3 | 0 | 4.71 | 0.10–0.05 |
| 10) LA32-1 | 10 | SS | 0 | 0 | 0 | 10 | – | | SS | 0 | 0 | 10 | – | |

**Table 4.** Designation of bands of activity, protomer composition, plant phenotype and genotype for the GOT-2 and GOT-3 isozymes found in *Vicia faba*

| GOT-2 | | | | | | | | GOT-3 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Band | Isozyme | Protomer composition | | | | | | Band | Isozyme | Protomer composition | | |
| 1 | Got-2a | (1)*$\alpha_{2a}\alpha_{2a}$ | $(\frac{1}{4})\alpha_{2a}\alpha_{2a}$ | $(\frac{1}{4})\alpha_{2a}\alpha_{2a}$ | | | | 1 | Got-3a | (1)$\alpha_{3a}\alpha_{3a}$ | $(\frac{1}{4})\alpha_{3a}\alpha_{3a}$ | |
| 2 | Got-2b | | $(\frac{1}{2})\alpha_{2a}\alpha_{2b}$ | | | | | 2 | Got-3b | | $(\frac{1}{2})\alpha_{3a}\alpha_{3b}$ | |
| 3 | Got-2c | | $(\frac{1}{4})\alpha_{2b}\alpha_{2b}$ | $(\frac{1}{2})\alpha_{2a}\alpha_{2c}$ | (1)$\alpha_{2b}\alpha_{2b}$ | $(\frac{1}{4})\alpha_{2b}\alpha_{2b}$ | | 3 | Got-3c | | $(\frac{1}{4})\alpha_{3b}\alpha_{3b}$ | (1)$\alpha_{3b}\alpha_{3b}$ |
| 4 | Got-2d | | | | | $(\frac{1}{2})\alpha_{2b}\alpha_{2c}$ | | | | | | |
| 5 | Got-2e | | | $(\frac{1}{4})\alpha_{2c}\alpha_{2c}$ | | $(\frac{1}{4})\alpha_{2c}\alpha_{2c}$ | (1)$\alpha_{2c}\alpha_{2c}$ | | | | | |
| Phenotype | | $F^FF^F$ | $F^FF$ | $F^FS$ | FF | FS | SS | | | FF | FS | SS |
| Genotype | | *Got-2a/ Got-2a* | *Got-2a/ Got-2b* | *Got-2a/ Got-2c* | *Got-2b/ Got-2b* | *Got-2b/ Got-2c* | *Got-2c/ Got-2c* | | | *Got-3a/ Got-3a* | *Got-3a/ Got-3b* | *Got-3b Got-3b* |

* In parenthesis is reported the relative proportion expected for the isozymes in each phenotype

Progeny analysis of selfed $S_0$ individuals (Table 3) showed that the FF phenotype in the GOT-2 zone bred true and produced only FF progenies. On the other hand, $S_0$ individuals showing the FS phenotype (Alto-2) produced FF, FS, and SS progenies in the ratio 1:2:1. Parental plants that showed the $F^FF$ phenotype (LA 11-1) produced a progeny with few $F^FF$ individuals, several FF individuals, and no $F^FF^F$ or some other phenotypes.

The observed positional variation of the bands within the GOT-2 zymograms of the analyzed progenies provided evidence that these bands were the sites of a group of dimeric isozymes encoded by three alleles at one locus. The locus has been designated *Got-2;* the three alleles have been indicated *Got-2a, Got-2b,* and *Got-2c,* and the subunits (protomers) they encoded $\alpha_{2a}$, $\alpha_{2b}$, $\alpha_{2c}$, respectively. The isozyme designation, the subunit composition corresponding to the bands made visible in the five possible GOT-2 phenotypes, and the allelic combination (genotype) in these phenotypes are reported in Table 4.

Phenotypes FF possessed the homozygous genotype *Got-2a/Got-2a* and were expected to breed true. The progenies of the 83C-1, Pam1-1a, Pam1-3a, 312-2, LA2-2, and LA16-1 parental individuals showed that this was the case.

The phenotype FS possessed the heterozygous genotype *Got-2b/Got-2c* and was expected to produce a segregating progeny with genotypes (and phenotypes) in the ratio of 1 *Got-2b/Got-2b* (FF) : 2 *Got-2b/Got-2c* (FS) : 1 *Got-2c/Got-2c* (SS). The progeny of the Alto-2 parental individual showed that this was the case.

The phenotype $F^FF$ possessed the heterozygous genotype *Got-2a/Got-2b* and was expected to produce a segregating progeny with the following genotype (and phenotype) ratio: 1 *Got-2a/Got-2a* ($F^FF^F$) : 2 *Got-2a/Got-2b* ($F^FF$) : 1 *Got-2b/Got-2b* (FF). The progeny of the LA11-1 $F^FF$ parental individual did not show genotypes according to that ratio and there was a lack of $F^FF^F$ individuals and an excess of FF individuals compared to the expected proportions. Selfing of the $F^FF^F$ and $F^FS$ parental

individuals of Fig. 1, whose genotype was assumed to be *Got-2a/Got-2a* and *Got-2a/Got-2c*, respectively, did not result in the production of selfed seeds. The above situation indicated that the *Got-2a* allele was probably strongly linked (1) to a gametophytic factor of the type described by Perry (1945), which reduced the competitive ability of the pollen tube carrying the *Got-2a* allele compared to pollen tube with other *Got-2* alleles, or (2) to a semilethal factor that lowered the viability of the $F^FF^F$, $F^FF$, and $F^FS$ individuals. The $F^FF^F$ and $F^FS$ individuals were very rare and were found only once in the population of parental plants. Therefore, the homozygous *Got-2a/Got-2a* and the heterozygous *Got-2a/Got-2c* genotype composition assumed for the $F^FF^F$ and $F^FS$ phenotypes needs further progeny testing in other *V. faba* populations. Phenotype SS breed true (LA32-1) and confirmed that they were expressed by the homozygous genotype *Got-2c/Got-2c*.

Three bands of isozyme activity were observed in the GOT-3 zone among the analyzed plants. The isozymes in band 1 were present alone in phenotype FF (Fig. 1 D) or together with the isozymes in bands 2 and 3 in phenotype FS (Fig. 1 D). The isozymes made visible in band 2 were always together with those in bands 1 and 3 in phenotype FS. The isozymes in band 3 were made visible alone in phenotype SS (Fig. 1 A–D) or together with those in band 1 or 2 in phenotype FS.

The FF phenotype was rare and it was found only in the progeny of FS individuals (Table 3). Therefore progeny analysis regarded only the selfed $S_0$ individuals whose phenotype was either FS or SS. The SS parental plants bred true and all their progeny showed the SS phenotype. $S_0$ individuals showing the FS phenotype (Gemini-1) produced a progeny with three FS and four FF individuals and none with SS individuals. This result was certainly due to the small progeny size observed, which was the consequence of unusual microenvironment created by the paper-glassine bag used in selfing the $S_0$ plants. A large progeny size should have also exhibited SS individuals.

Despite the small progeny size available in certain cases, the observed positional variation of the bands within the GOT-3 zymograms of the analyzed progenies provided evidence that these bands were the sites of a group of dimeric isozymes encoded by two alleles at one locus. The locus has been designated *Got-3;* the two alleles have been called *Got-3a* and *Got-3b* and the subunits (protomers) they encoded $\alpha_{3a}$ (the protomers composing the fastest isozymes) and $\alpha_{3b}$ (the protomers composing the slower isozymes), respectively. The isozyme designation, the subunit composition corresponding to the bands made visible in the three possible phenotypes, and the allelic combination (genotype) in these phenotypes are reported in Table 4.

From the segregation observed in the progeny of FS phenotype it was inferred that: (a) phenotype FF possessed the homozygous genotype *Got-3a/Got-3a*, (b) the FS phenotype was expressed by the heterozygous genotype *Got-3a/Got-3b*, and (c) the phenotype SS possessed the homozygous genotype *Got-3b/Got-3b* and as expected, bred true.

*Superoxide dismutase, SOD*

Three zones of activities were observed in the SOD zymograms (Fig. 2). The isozymes in zone 1 (the anodal zone) were designated SOD-1 and in the analyzed parental plants they were made visible in three bands. Band 1 was either alone (phenotype FF in Fig. 2 A–C) or together with bands 2 and 3 (phenotype FS in Fig. 2A). However when band 1 was alone, it was always coupled with a minor band in anodal position, which may have been due to conformational isozyme variants of SOD or oxidoreductase enzymes that were coordinately regulated with SOD. Band 2 was always together with bands 1 and 3 (phenotype FS, Fig. 2 A) and showed double negative staining intensity compared to that of the other two bands. Band 3 was either alone (phenotype SS, Fig. 2 A) or together with bands 1 and 2 (phenotype FS). When band 3 was alone, it was always coupled with a minor band in anodal position (as was the case for band 1 in phenotype FF), whose presence may have been due to the same cause as that of the minor band made visible together with band 1.

In zone 2 (the intermediate zone), called SOD-2, the isozymes of the studied progenies were made visible in three bands. Band 1 was shown to be either alone (phenotype FF, Fig. 2 B) or together with bands 2 and 3 (phenotype FS, Fig. 2 B). Band 2 was always found together with bands 1 and 3 (phenotype FS). Band 3 was visualized either alone (phenotype SS, Fig. 2 A–C) or together with bands 1 and 2 (phenotype FS).

In zone 3 (the cathodal zone), designated SOD-3, the isozymes of the studied progenies were made visible in five bands. These bands were fainter than those observed in the other two zones. Bands 1 and 5 were observed alone in phenotype FF and SS, respectively, or together with the other four bands in phenotype FS (Fig. 2 C).

When gels were stained in the presence of 2 m*M* KCN (Fig. 3 A), the SOD activity in zone 1 was inhibited, the SOD activity in zone 2 appeared to be almost nil, and the SOD activity of zone 3 was not inhibited. Fridovich (1975) and Foster and Edwards (1980) found that only copper- and zinc-containing SOD in eukaryotic cytosol and chloroplasts were reversibly inhibited by a millimolar concentration of cyanide, while manganese-containing SOD, found in mitochondria and prokaryotes, were not inhibited by cyanide. Therefore on the basis of this indication and of the KCN experiment, it is

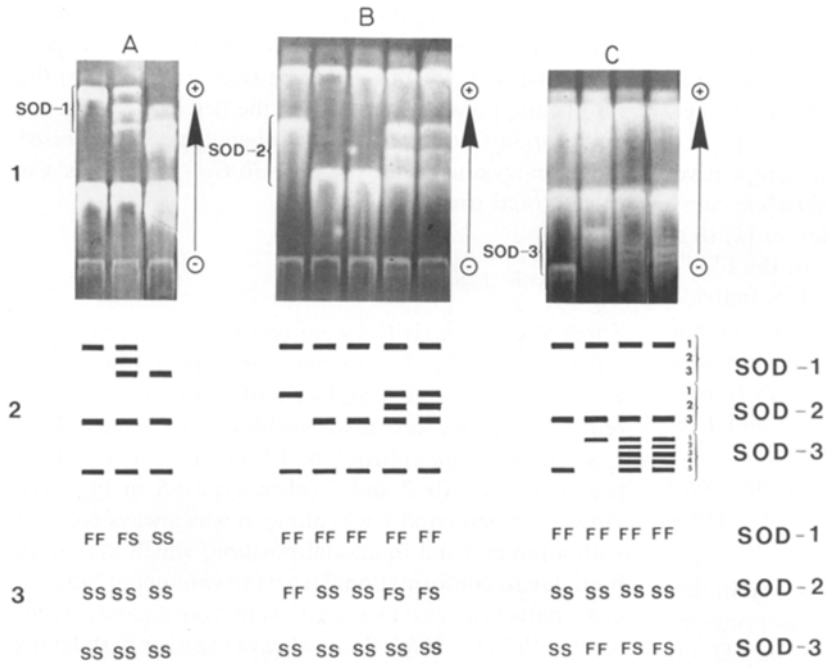


**Fig. 2A–C.** Zymogram *1*, drawing of the bands of isozyme activity *2*, and phenotype code *3* for SOD from leaf extract of different groups of plants **A–C** of *Vicia faba*. *Arrow* denotes the direction of migration in the gel; + and − denote anode and cathode, respectively

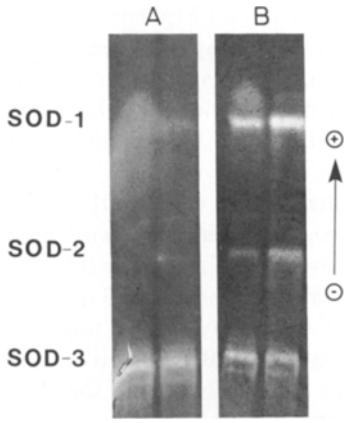


**Fig. 3A and B.** SOD zymogram phenotypes obtained **A** in the presence and **B** absence of 2 m*M* KCN in the staining solution. *Arrow* denotes the direction of migration in the gel; + and − denote anode and cathode, respectively

suggested that: (1) the isozymes in the SOD-1 and SOD-2 zones were copper- and zinc-containing SODs and the isozymes made visible in the SOD-3 zone were manganese-containing SODs; (2) SOD-1, SOD-2, and SOD-3 were the chloroplastic, the cytosol, and the mitochondrial SOD isozymes, respectively.

Progeny analysis of selfed $S_0$ individuals (Table 5) showed that: (1) in the SOD-1, SOD-2, and SOD-3 zones the FF and SS phenotypes bred true, and (2) that FS phenotypes for the SOD-1 and SOD-2 zones produced FF, FS, and SS phenotypes in a ratio that was significantly different from 1:2:1 at the 0.01 probability level in only one case (progeny LA62-2).

The observed variation in the number of SOD bands in the three zones and the variability observed in progenies of triple-banded phenotypes provided evidence of the following isozyme composition and genetical determination of those bands (Table 6).

SOD-1 isozymes were dimeric and were composed by protomers encoded by two alleles at a locus named *Sod-1*. The two alleles, *Sod-1a* and *Sod-1b*, encoded the protomers $\sigma_{1a}$ and $\sigma_{1b}$, respectively, in equal quantity. Homozygous *Sod-1a*/*Sod-1a* genotypes (VT1-3a, LA1-1a, and LA4-2) produced only the dimeric Sod-1a isozymes whose protomer composition was $\sigma_{1a}\,\sigma_{1a}$ which were made visible in band 1. Heterozygous *Sod-1a*/*Sod-1b* genotypes (LA11-1, LA40-2, LA28BA-1, and LA63-2) produced the dimeric Sod-1a, Sod-1b, and Sod-1c isozymes which appeared after all possible combinations of the $\sigma_{1a}$ and $\sigma_{1b}$ protomers coded by the *Sod-1a* and *Sod-1b* alleles, respectively; the relative proportion of these isozymes was obtained using the binomial formula $(\frac{1}{2}\sigma_{1a}+\frac{1}{2}\sigma_{1b})^2$ and was made visible in bands 1, 2, and 3. Homozygous *Sod-1b*/*Sod-1b* genotypes (Alto-2) produced only the dimeric Sod-1c isozymes whose protomer composition was $\sigma_{1b}\,\sigma_{1b}$ which were made visible in band 3.

SOD-2 isozymes were dimeric and were composed by protomers encoded by two alleles at a locus designated *Sod-2*. The two alleles, *Sod-2a* and *Sod-2b*, encoded the protomers $\sigma_{2a}+\sigma_{2b}$, respectively, in equal quantity. Homozygous *Sod-2a*/*Sod-2a* parental plants with zymogram phenotype FF were not identified, but they were

**Table 5.** Phenotypic frequencies observed in the zymogram of SOD-1, SOD-2, and SOD-3 isozyme systems in $S_1$ progenies of *Vicia faba* L. $S_0$ parental plants

| Parental plant ($S_0$) | No. plants in $S_1$ progeny | SOD-1 | | | | $\chi^2$ | $P$ | SOD-2 | | | | $\chi^2$ | $P$ | SOD-3 | | | | $\chi^2$ | $P$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $S_0$ | $S_1$ | | | | | $S_0$ | $S_1$ | | | | | $S_0$ | $S_1$ | | | | |
| | | | FF | FS | SS | | | | FF | FS | SS | | | | FF | FS | SS | | |
| 1) ALTO-2 | 55 | SS | 0 | 0 | 55 | – | | FS | 16 | 34 | 5 | 7.47 | 0.025–0.010 | SS | 0 | 0 | 55 | – | |
| 2) VT1-3a | 10 | FF | 10 | 0 | 0 | – | | SS | 0 | 0 | 10 | – | | SS | 0 | 0 | 10 | – | |
| 3) LA1-1 | 14 | FF | 14 | 0 | 0 | – | | SS | 0 | 0 | 14 | – | | SS | 0 | 0 | 14 | – | |
| 4) LA4-2 | 16 | FF | 16 | 0 | 0 | – | | SS | 0 | 0 | 16 | – | | FS | 3 | 7 | 6 | 1.37 | 0.75–0.50 |
| 5) LA11-1 | 33 | FS | 5 | 25 | 3 | 9.00 | 0.025–0.010 | SS | 0 | 0 | 33 | – | | SS | 0 | 0 | 33 | – | |
| 6) LA40-2 | 9 | FS | 2 | 6 | 1 | 1.20 | 0.75 –0.50 | SS | 0 | 0 | 9 | – | | SS | 0 | 0 | 9 | – | |
| 7) LA28BA-1 | 14 | FS | 4 | 8 | 2 | 0.85 | 0.75 –0.50 | SS | 0 | 0 | 14 | – | | SS | 0 | 0 | 14 | – | |
| 8) LA63-2 | 17 | FS | 10 | 6 | 1 | 11.00 | < 0.005 | SS | 0 | 0 | 17 | – | | SS | 0 | 0 | 17 | – | |

**Table 6.** Designation of bands of activity, protomer composition, plant phenotype and genotype for the SOD-1, SOD-2, and SOD-3 isozymes found in *Vicia faba*

| SOD-1 | | | | | SOD-2 | | | | | SOD-3 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Band | Isozyme | Protomer composition | | | Band | Isozyme | Protomer composition | | | Band | Isozyme | Protomer composition | | |
| 1 | Sod-1a | (1)* $\sigma_{1a}\sigma_{1a}$ | ($\frac{1}{4}$) $\sigma_{1a}\sigma_{1a}$ | | 1 | Sod-2a | (1) $\sigma_{2a}\sigma_{2a}$ | ($\frac{1}{4}$) $\sigma_{2a}\sigma_{2a}$ | | 1 | Sod-3a | (1) $\sigma_{3a}\sigma_{3a}\sigma_{3a}\sigma_{3a}$ | (1/16) $\sigma_{3a}\sigma_{3a}\sigma_{3a}\sigma_{3a}$ | |
| 2 | Sod-1b | | ($\frac{1}{2}$) $\sigma_{1a}\sigma_{1b}$ | | 2 | Sod-2b | | ($\frac{1}{2}$) $\sigma_{2a}\sigma_{2b}$ | | 2 | Sod-3b | | (4/16) $\sigma_{3a}\sigma_{3a}\sigma_{3a}\sigma_{3b}$ | |
| 3 | Sod-1c | | ($\frac{1}{4}$) $\sigma_{1b}\sigma_{1b}$ | (1) $\sigma_{1b}\sigma_{1b}$ | 3 | Sod-2c | | ($\frac{1}{4}$) $\sigma_{2b}\sigma_{2b}$ | (1) $\sigma_{2b}\sigma_{2b}$ | 3 | Sod-3c | | (6/16) $\sigma_{3a}\sigma_{3a}\sigma_{3b}\sigma_{3b}$ | |
| | | | | | | | | | | 4 | Sod-3d | | (4/16) $\sigma_{3a}\sigma_{3b}\sigma_{3b}\sigma_{3b}$ | |
| | | | | | | | | | | 5 | Sod-3e | | (1/16) $\sigma_{3b}\sigma_{3b}\sigma_{3b}\sigma_{3b}$ | (1) $\sigma_{3b}\sigma_{3b}\sigma_{3b}\sigma_{3b}$ |
| Phenotype | | FF | FS | SS | | | FF | FS | SS | | | FF | FS | SS |
| Genotype | | *Sod-1a/ Sod-1a* | *Sod-1a/ Sod-1b* | *Sod-1b/ Sod-1b* | | | *Sod-2a/ Sod-2a* | *Sod-2a/ Sod-2b* | *Sod-2b/ Sod-2b* | | | *Sod-3a/ Sod-3a* | *Sod-3a/ Sod-3b* | *Sod-3b/ Sod-3b* |

* In parenthesis is reported the relative proportion expected for the isozymes in each phenotype

expected to produce only the dimeric Sod-2a isozymes made visible in band 1, whose protomer composition should have been $\sigma_{2a}$ $\sigma_{2a}$. Heterozygous *Sod-2a/Sod-2b* parental plants (Alto-2) produced the dimeric Sod-2a, Sod-2b, and Sod-2c isozymes which appeared after all possible combinations of the $\sigma_{2a}$ and $\sigma_{2b}$ protomers coded by the *Sod-2a* and *Sod-2b* alleles, respectively; these isozymes were made visible in bands 1, 2, and 3. The progeny of the heterozygous parental plant showed FF, FS, and SS phenotypes but with a deficiency of the SS phenotype compared to the expected 1 FF : 2 FS : 1 SS ratio. The cause of this distorted segregation ratio could have been sampling effects, linkage of the *Sod-2b* allele with gametophytic factor, and semilethality of the *Sod-2a* allele. However, the good fertility of the SS parental phenotypes, which were homozygous *Sod-2b/Sod-2b* genotypes, excluded the possibility that the *Sod-2a* allele was semilethal. Therefore, sampling effect or linkage to gametophytic factors may be the cause of the distorted phenotypic ratio. The homozygous *Sod-2b/Sod-2b* parental genotypes (VT1-3a, LA1-1a, LA4-2, LA11-1, LA40-2, LA28BA-1, and LA 63-2) produced only the dimeric Sod-2c isozymes whose protomer composition was $\sigma_{2b}$ $\sigma_{2b}$, which were made visible in band 3.

SOD-3 isozymes were tetrameric and were composed by protomers encoded by two alleles at a locus called *Sod-3*. The two alleles, *Sod-3a* and *Sod-3b,* encoded the protomers $\sigma_{3a}$ and $\sigma_{3b}$, respectively, in equal quantity. Homozygous *Sod-3a/Sod-3a* parental genotypes showing the zymogram phenotype FF were not identified, probably due to the rarity of the *Sod-3a* allele. However, such genotypes were expected to produce only the tetrameric Sod-3a isozymes made visible in band 1, whose protomer composition should be $\sigma_{3a}\sigma_{3a}\sigma_{3a}\sigma_{3a}$. Although absent among the parental phenotypes analyzed, the homozygous *Sod-3a/Sod-3a* genotypes showing zymogram phenotype FF were found in the progeny of FS parental phenotypes. As matter of fact, the FS parental phenotype LA4-2 had the heterozygous genotype *Sod-3a/Sod-3b,* which produced a progeny with homozygous genotype *Sod-3a/Sod-3a* and *Sod-3b/Sod-3b* (showing only the tetrameric isozymes Sod-3a and Sod-3e, respectively), plus the heterozygous genotype *Sod-3a/Sod-3b*. This heterozygous genotype produced the five isozymes Sod-3a, Sod-3b, Sod-3c, Sod-3d, and Sod-3e made visible in bands 1 through 5, whose expected quantitative proportion was obtained by using the expression $(\frac{1}{2}\sigma_{3a} + \frac{1}{2}\sigma_{3b})^4$, where $\sigma_{3a}$ and $\sigma_{3b}$ indicated the protomer encoded by the *Sod-3a* and *Sod-3b* allele, respectively. The expected relative amount of Sod-3a and Sod-3b isozymes in the FS phenotype was not sufficient to allow their visibility in the FS zymogram (expecially for Sod-3a isozymes). The progeny of the heterozygous parental genotypes deviated by the expected 1 FF : 2 FS : 1 SS phenotypical ratio because of sampling effect due to the small progeny size.

Homozygous *Sod-3b/Sod-3b* parental genotypes showing the zymogram phenotype SS were very frequent and bred true.

*Esterase, EST*

The plant samples in the five open-pollinated faba bean varieties gave an esterase zymogram from crude extracts of young leaves of 3 to 4-week-old plants that was composed of at least eight zones of activities for a total of ten bands. The banding pattern of parental plants and selfed progenies were not always related in most of the zones except for the fast-moving EST isozymes identified in the three most anodal zones (Fig. 4). These zones have been designated EST-1, EST-2, and EST-3.

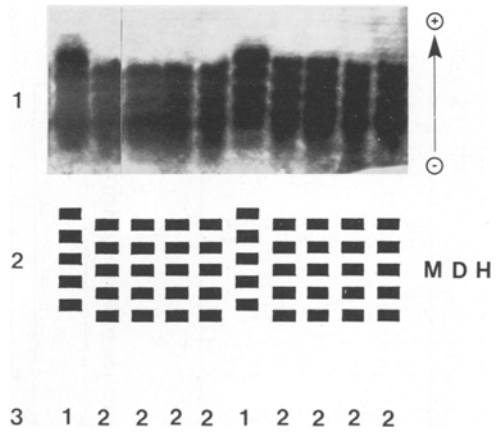


**Fig. 4.** Zymogram *1*, drawing of the bands of isozyme activity *2*, and phenotype code *3* for EST from leaf extract of different plants of *Vicia faba*. The numbers in the zymogram pattern *1* indicate the bands analogous to those reported in the band drawing section *2*. *Arrow* denotes the direction of migration in the gel; + and − denote anode and cathode, respectively

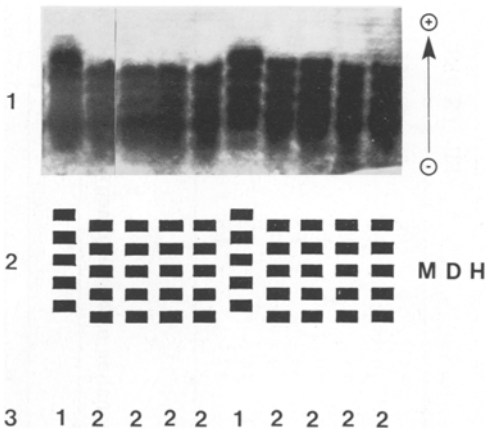


**Fig. 5.** Zymogram *1*, drawing of the bands of isozyme activity *2*, and phenotype code *3* for MDH from leaf extract of different plants of *Vicia faba*. *Arrow* denotes the direction of migration in the gel; + and − denote anode and cathode, respectively

The zone EST-1 was composed of the fastest-moving EST isozymes, and all individuals analyzed showed the same isozyme type which was made visible in a monomorphic band.

In the EST-2 zone there were two bands of activity: one showing isozymes a bit slower than that in the Est-1 zone (Est-2a isozymes) and another with isozymes adjacent to those of the Est-3 zone (Est-2b isozymes). Individuals showed only Est-2a or Est-2b isozymes or both.

In EST-3 zone there were also two bands of isozyme activity (Est-3a and Est-3b) and individuals showed only one of the bands (Est-3a or Est-3b) or both.

Based on the zymogram patterns from parental individuals and some of their progenies, it has been hypothesized that the fastest-moving esterase isozymes were each produced by codominant alleles at different loci. Unfortunately, the visibility of the isozymes in progenies of double-banded phenotypes (FS) in zones EST-2 and EST-3 was not always clear and no exact segregation ratios can be given.

*Malate dehydrogenase, MDH*

Two MDH zymogram types have been made visible by starch gel in the same individuals surveyed for EST (Fig. 5). Each zymogram type showed five bands of MDH isozyme activity, which was evidence that more than one locus is involved in the production of MDH isozymes. The five bands in the MDH zymogram type 1 were slightly anodally advanced compared to those of zymogram type 2. The individuals showing MDH zymogram type 1 or 2 bred true, indicating that each individual possessed fixed alleles at the loci controlling MDH isozymes.

## Discussion

Many workers have used tissue from *Vicia faba* plants for enzyme studies (Hill-Cottingham 1983). The use of isozyme genes as markers in the genetic studies of *Vicia faba* have been discussed by Peat and Adham (1984). However, the genetics of the *Vicia faba* isozymes have not been worked out extensively and only limited information is available on the genetic control of few isozymes.

GOT isozymes were studied by Yamamoto et al. (1982) to compare isozyme polymorphism in species of the section *Faba*, and a four-banded pattern for all the *V. faba* strain studied was reported.

A genetic control based on two alleles at one locus has been proposed by Suso and Moreno (1982) for the fast-moving GOT-B isozymes. These GOT-B isozymes apparently correspond to the GOT-1 isozymes reported in this paper, for which no variation has been found in the zymogram pattern of the analyzed materials. Gates and Boulter (1979) studied the GOT patterns from cotyledon extracts of *V. faba* inbred lines and $F_1$ hybrids, and their pattern was not comparable with those reported here.

On the basis of a model explaining the variability observed in the zymogram of parental plants and selfed progenies analyzed in this paper, it has been shown that in *Vicia faba* the isozymes in GOT-2 and GOT-3 zones are under the control of *Got-2* and *Got-3* loci, respectively. These two loci showed allelic variability, and three alleles have been found for the *Got-2* locus and two alleles at the *Got-3* locus. This situation creates two new isozyme marker loci, the *Got-2* and *Got-3* loci.

If the GOT-1 zone indicated in this paper corresponds to the GOT-B zone of Suso and Moreno (1982), then the locus controlling GOT-1 isozymes, which can be called *Got-1*, represents another potential isozyme marker locus.

Three loci have been found to control the expression of GOT isozymes in lentil (*Lens culinaris*) (Zamir and Ladizinsky 1984). In soybeans (*Glycine max* [L.] Merrill), at least three GOT loci controlling the isozyme production in each subcellular activity site (plastid, mitochondrial, and cytosol) have been hypothesized (Kiang and Gorman 1983). Three loci have been also found to control GOT isozymes in pea (Weeden and Marx 1987). These loci have been located on chromosome 1, 2, and 3 of pea. Apparently in the material analyzed in this paper, there was an independent segregation of GOT-2 and GOT-3 phenotypes, and if the chromosome-arm locations of the *Got* genes in *Leguminosae* is conserved, it may be hypothesized that in faba bean the GOT isozymes are controlled by three independent genes, as is the case in pea.

The *Vicia faba* GOT isozymes are dimeric as has been found in *Lycopersicon* (Rick 1983), *Capsicum* (McLeod et al. 1986), *Triticinae* (Hart and Langston 1977), and *Pinus* (Rudin 1975).

The ascertained cellular compartmetalization of SOD isozymes (in the sense that SOD-1, SOD-2, and SOD-3 were the chloroplastic, the cytosol, and the mitochondrial SOD isozymes), and the observation that SOD-1 and SOD-2, were dimeric isozymes and SOD-3 were tetrameric isozymes, agree with what is known in other organisms (Fridovich 1975), and in particular in plant species such as maize (Baum and Scandalios 1982) and *Triticinae* species (Jaaska 1982). However, there are differences in the pattern of SOD-3 inheritance observed in maize and what has been detected in faba bean plants. In maize, the mitochondrial SOD-3 isozymes were visualized in a five-banded pattern which did not segregated upon self-fertilization; this was taken as evidence of both a tetrameric structure and an inheritance controlled by duplicate genes (Baum and Scandalios 1982). In faba

bean, the five-banded pattern segregated in the progeny upon self-fertilization and the tetrameric structure were under the control of two alleles at one locus.

Multiple forms of EST isozymes have been found previously in *Vicia faba*. A total of 10 different bands were observed for the EST isozyme system in 40 broad bean cultivars by Bassiri and Rouhani (1977); Gates and Boulter (1979) described a total of 18 EST bands in inbred lines; the number present in any one line ranged from 5–13. Peat and Adham (1984) reported finding a total of 26 bands in the zymogram pattern for esterase from nearly expanded leaves of plants from a wide range of *V. faba* cultivars. The genetic control for the esterase isozymes have been reported only for the eight most cathodal isozymes by Peat and Adham (1984), and three Mendelian genes with codominant alleles have been ascertained to code for these isozymes. In the analysis reported here, there was evidence of a genetic control due to Mendelian genes with codominant alleles also for the most anodal EST-1, EST-2, and EST-3 isozymes. However, differences in band staining intensity among the isozymes of these three zones in the progenies resulted in zymograms not always easily interpretable, which prevented the estimation of segregation ratios in the progenies.

For MDH, three or four isozymes have been reported in many higher plants (Gottlieb 1982). Distinctly different forms of MDH have been found in the cytosol, microbodies, and mitochondria (Newton 1983). Usually in a zymogram pattern, the most cathodal MDH isozymes are the microbody forms and the most anodal are either the cytosolic or the mitochondrial MDH forms. In maize only the anodal and mitochondrial MDH isozymes showed variants and these were coded by nuclear genes (Scandalios 1969; Longo and Scandalios 1969) which followed a strict Mendelian inheritance due to three unlinked structural genes (Goodman et al. 1980).

In the MDH zymogram pattern reported here for faba bean, it seems that only the most anodal MDH isozymes showed variants, as in maize, while the other four MDH isozymes seem to be invariant. Also in faba bean, the most anodal MDH isozymes are probably the mitochondrial form; however, their mode of inheritance will be difficult to assess unless proper crosses between cross-compatible individuals with different anodal MDH phenotypes can be performed.

In conclusion, the reported results, although not interpretable in the strict Mendelian sense in all cases due to the small sample size of certain selfed progenies, were evidence that the GOT-2, GOT-3, SOD-1, SOD-2, and SOD-3 isozymes were each under the control of one locus. Therefore, these five loci (*Got-2, Got-3, Sod-1, Sod-2,* and *Sod-3*) can be considered new useful isozyme gene markers to be used in *Vicia faba* breeding for linkage study, varietal fingerprinting, outcrossing rate estimate, and indirect selection for quantitative characters. There was also indirect evidence that GOT-1, EST-1, EST-2, and EST-3 were each under the control of one locus. There was phenotypic variability for MDH isozymes, but a genetical interpretation of the observed variability was not possible on the basis of the analysis of selfed progenies. Efforts are being carried out to search divergent isozyme phenotypes in the same botanical group in order to analyze $F_1$ and $F_2$ progenies.

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