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ACCUMULATION OF STEROLS AND STEROIDAL SAPOGENINS IN DEVELOPING FENUGREEK PODS: POSSIBLE BIOSYNTHESIS *IN SITU*

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Key Word Index—*Trigonella foenum-graecum*; Leguminosae; fenugreek; pods; accumulation; biosynthesis; sterols; steroidal sapogenins.

Abstract—The accumulation of sterols and steroidal sapogenins was investigated in the seeds and pericarps of fenugreek (*Trigonella foenum-graecum* L.) during the development and maturation of the pod. Flowers and young pods contained high levels of these substances which decreased during the development of pericarps and seeds. Steroidal sapogenins (mainly diosgenin, tigogenin and their C-25 epimers) then accumulated strongly in seeds while the increase of sterols was very slight. Cholesterol showed similar, but less dramatic changes. Several other sterols, including pollinastanol (14α-methyl-9β,19-cyclo-5α-cholestan-3β-ol), and dihydroxylated sapogenins also appeared during the development and maturation of seeds. Labelling experiments with [2-^{14}C]-acetate were carried out on excised maturing pods. Radioactivity was found in the four sterolic forms, mono- and dihydroxylated sapogenins from the seeds. Labelled diosgenin was isolated and purified to constant specific radioactivity. These results indicate that excised maturing pods of fenugreek can biosynthesize sterols and steroidal sapogenins.

INTRODUCTION

Steroidal sapogenins are secondary metabolites whose biosynthetic precursors are sterols, especially cholesterol [1]. They are mainly found as glycosides called steroidal saponins. Although steroidal sapogenins have been almost exclusively isolated from plants [2], very few plants accumulate these compounds in their seeds. Fenugreek (*Trigonella foenum-graecum* L.) seeds have been recognized for a long time as a potential source of diosgenin ((25*R*)-spirost-5-en-3β-ol) [3]. This steroidal sapogenin is considered a basic compound in the hemisynthesis of steroid drugs such as cortisone and sexual hormones. It is extracted mainly from Dioscoreaceae tubers.

Despite their economic importance, very little is known about the biosynthesis or the accumulation processes of such compounds in seeds or other organs (tubers of Dioscoreaceae). A translocation of diosgenin from the vegetative parts to the seeds was postulated in *Costus speciosus* K. Sm. [4, 5]. However, biosynthesis of sterols, steryl glycosides and acylsteryl glycosides has been reported in immature pea seeds [6, 7]. Eichenberger [8] showed the possible involvement of the cholesterol and its derivatives in the biosynthesis of nuatigenin and the corresponding saponins of oat. Fenugreek seeds contain a higher level of cholesterol than other plants [9] and important amounts of steroidal sapogenins. This paper presents investigations on the accumulation of both sterols and steroidal sapogenins in the pericarps and seeds of fenugreek pods during their development and maturation. Complementary, labelling experiments demonstrated the biosynthesis of sterols and steroidal sapogenins in excised pods.

RESULTS AND DISCUSSION

During the first part of pod development, the accumulation of fresh and dry matter was greater in the pericarps than in the seeds (Fig. 1). From 48 to 66 days after anthesis (DAA), the growth of the seeds was more rapid and a maximum of fresh (Fig. 1A) and dry (Fig. 1B) weight was reached after 66 and 80 DAA, respectively. Seeds then accounted for 67.6% of the fresh weight and 74.9% of the dry weight of the pod. The last phase included the specific features associated with the cessation of growth and the onset of seed maturation. Physiological maturity, as indicated in soybean by maximum dry weight, relative water content $< 60\%$ and loss of green colour [10, 11], occurred in seeds at 76 DAA and slightly before in pericarps.

Sterol and steroidal sapogenin content was always higher in the seeds than in the pericarps of a pod (Fig. 2A and 2B). Accumulation in seeds of these metabolites occurred slowly until 60 DAA. The amount of steroidal

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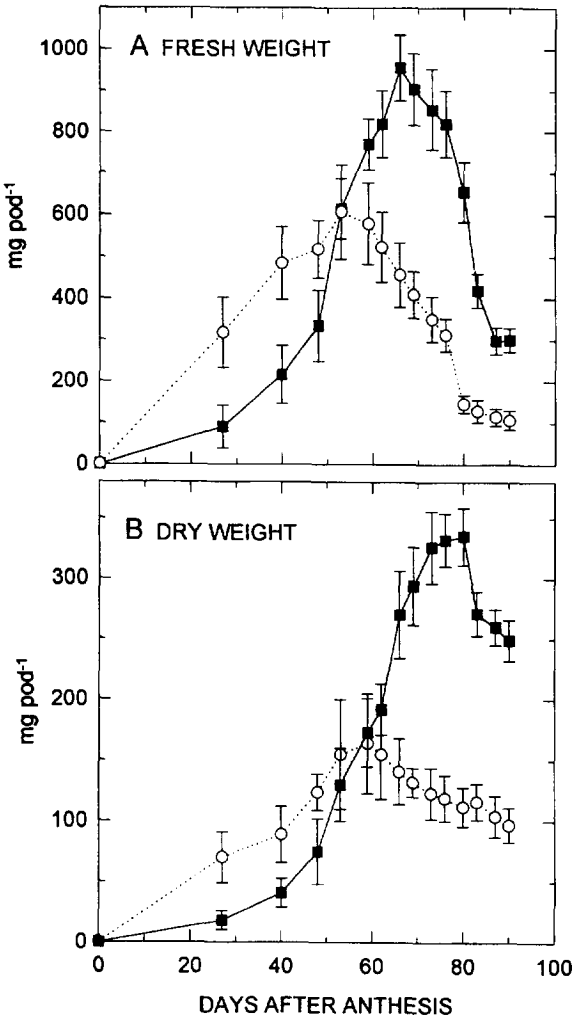


Fig. 1. Growth of seeds (■) and pericarps (○) during pod development. (A) Fresh weight; (B) dry weight. Values represent the mean ± s.e. of 20 determinations. Each pod contains 12–15 seeds.

sapogenins then increased more rapidly. At maximum, it was about five times higher than that of sterols. A slight decrease was detectable at the end of maturation. The pattern of accumulation of sterols and steroidal sapogenins was similar to those of fresh and dry matter (Fig. 1), of protein nitrogen [12] and of neutral lipids (data not given). On a dry weight basis, sterols (Fig. 2C) and steroidal sapogenins (Fig. 2D) showed similar values in the flowers and the first pods. These values declined in seeds and pericarps until 48 DAA. Sterols and steroidal sapogenins of the pericarps then continued to decrease slowly until the end of maturation. In seeds, sterols increased slightly while steroidal sapogenins accumulated dramatically (a nine-fold increase from 48 to 76 DAA). Free sterols and steryl esters were predominant over steryl glycosides and acylsteryl glycosides throughout pod development (Table 1). In the flowers and the first pods, free sterols and steryl esters accounted for 83–92% of total sterols (49.4–80% free sterols). Sterol conjugates then showed opposite changes in the pericarps and in the seeds. In pericarps, steryl glycosides and acylsteryl glycosides increased from 18.6 to 40.4% until 76 DAA and then declined to 32.4%. In seeds, these forms decreased from 10.9 to 6.3% until 76 DAA and increased at the two last stages to 24.8%. High levels in total and free sterols have been reported in the early stages of growth of soybean seeds [13] and maize kernels [14]. A decrease of the capacity for sterol biosynthesis has been observed during the development of pea seed [6]. A high rate of sterol biosynthesis occurs in actively growing and differentiating tissues [15–18]. During maize seed development, the highest activities of HMG-CoA reductase, a key enzyme in the biosynthesis of sterols and other isoprenoids, were found during stages of rapid mitotic divisions [19]. Free sterols have been assumed to play a role in the stabilization of membranes of barley [20]. Sterols of membrane fractions of maize coleoptiles are mainly in the free form [21]. Steryl esters, steryl glycosides and acylsteryl glycosidea are also present but their function in membranes requires further consideration. Thus, the high levels of total and free sterols found in the first stages of pod growth could be related to high rates of sterol biosynthesis occurring in young tissues, when the rates of mitosis and membrane formation are maximum. Tissues of flowers and small pods also contained high levels of total steroidal sapogenins. Similar observations have been reported on *C. speciosus* [4]. Diosgenin, the main steroidal sapogenin of this plant, accumulates in large amounts in the flowers before being transferred to the seeds.

The major sterols detected during pod development were the typical plant sterols (Fig. 3), sitosterol, 24-methyl cholesterol, cholesterol and stigmasterol, and other less common sterols, Δ7-cholesterol, Δ5-avenasterol and pollinastanol (14α-methyl-9β,19-cyclo-5α-cholestan-3β-ol). In comparison with other higher plants, fenugreek pod contains less stigmasterol while the amount of cholesterol is greater. To our knowledge, fenugreek are the only seeds in which pollinastanol has been reported since its presence was established by GC-MS [9] and ^{1}HNMR spectroscopy (J. Artaud, personal communication). It had never been described previously in plant tissues other than pollens [22, 23]. The composition in the early stages (flowers and small pods, Fig. 3) was characterized by a high proportion of cholesterol (7–9% of total sterols) and by the presence of stigmasterol and pollinastanol which were not detected in the first stages of pericarps and seeds. At the beginning of pod development, pericarps and seeds showed a simple composition but several minor sterols appeared during the next stages. In pericarps, Δ7-cholesterol, stigmasterol, pollinastanol and an unknown sterol were successively detected from 62 to 69 DAA. Their proportion increased until the end of maturation while those of cholesterol and sitosterol decreased. In seeds, the unknown sterol appeared from 40 DAA and its proportion increased, in the same manner as cholesterol and Δ7-cholesterol, until 69 DAA. The amounts of these three sterols then declined; pollinastanol appeared and sitosterol increased. As

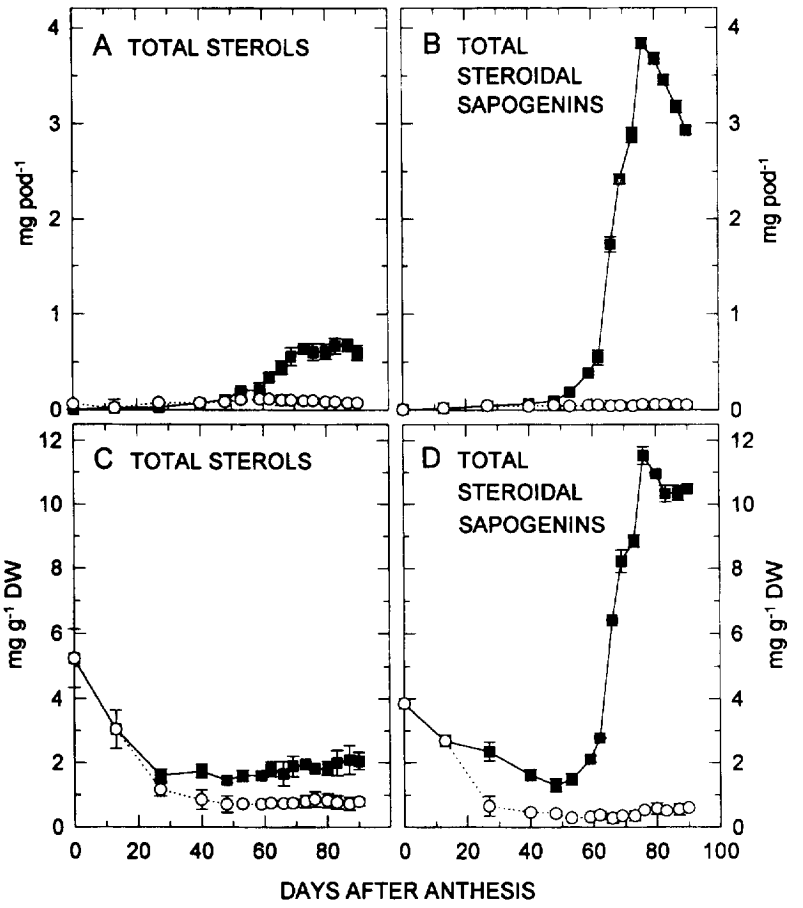


Fig. 2. Changes in sterols (A, C) and steroidal sapogenins (B, D) of seeds (■) and pericarps (○) during pod development. Values expressed per pod (A, B) and on a dry weight basis (C, D). Results represent the mean ± s.e. of three to six determinations.

previously demonstrated, pollinastanol can be involved in a particulate biosynthesis pathway of cholesterol [9, 24]. The respective changes of these two compounds support this hypothesis. Aging of tissues has been reported to be associated with modifications in the sterol composition. A decrease in the sitosterol:stigmasterol ratio was observed with aging of tissues in etiolated mung bean seedlings [25, 26] and in barley membranes [27]. Such a modification was not perceptible in pods. However, investigations on the vegetative parts of fenugreek plants revealed that stigmasterol is present at a higher level (1.4–6.8%) in roots, stems and leaves throughout development and maturation, and increases (to 9.8–18.5%) at the last stage, when tissues are totally dry.

Fenugreek seed steroidal sapogenins are structurally related. The 10 compounds identified differ only by the presence or absence of a double bond at C-5 and by the position in 5α or 5β of the hydrogen atom resulting from the reduction of this double bond, by the presence or the absence of a second hydroxyl group at C-2 (2α) and by the conformational position in *R* or *S* of the methyl group at C-25. First stages and pericarps showed similar compositions and few changes were observed during the maturation (Fig. 4). Smilagenin ((25*R*)-5β-spirostan-3β-ol) and sarsasapogenin, its 25-*S* epimer, were predominant throughout the development of the pericarps. In the seeds, diosgenin was the most abundant sapogenin at all stages and contributed, to a large degree, to the increase in total sapogenins. Diosgenin and its 25-*S* epimer, yamogenin (not separated from tigogenin ((25*R*)-5α-spirostan-3β-ol) in our analysis), accounted for 97.2% of total sapogenins in newly formed seeds (27 DAA). These three sapogenins then declined to 66.1% until 69 DAA and remained between 69.2 and 72.2% until the end of maturation. Smilagenin and sarsasapogenin were present at lower levels than in pericarps. Seeds were also characterized by the presence of dihydroxylated sapogenins: yuccagenin ((25*R*)-spirost-5-en-2α,3β-diol) and its 25-*S* epimer, lilagenin, gitogenin ((25*R*)-5α-spirostan-2α,3β-diol) and its 25-*S* epimer, neogitogenin. These compounds were absent in the first stages and in the pericarps. They were detected in seeds from 40 DAA and accumulated until the end of maturation. In addition to these results, the presence of a very low level of spirosta-3,5-diens proved the efficiency of our conditions of hydrolysis. These products are formed under too-drastic acid hydrolysis by loss of the hydroxyl group at C-3 of sapogenins possessing a double bond between C-5 and

Table 1. Changes in sterol conjugates during pod development

Days after anthesis	Stages	Steryl glycosides	Acylsteryl glycosides	Free sterols	Steryl esters
		(% of total sterols)			
0	Flowers	12.7 ± 1.4	3.5 ± 0.9	49.4 ± 2.5	34.4 ± 1.8
13	Small pods	4.4 ± 1.2	3.6 ± 1.2	80.0 ± 2.0	12.0 ± 1.6
27	Pericarps	5.6 ± 0.8	13.0 ± 1.8	51.7 ± 2.7	29.7 ± 1.6
40		8.4 ± 0.5	14.2 ± 0.9	48.3 ± 1.1	29.1 ± 1.1
53		6.8 ± 0.5	12.4 ± 1.7	44.4 ± 2.1	36.4 ± 0.7
62		11.2 ± 0.5	12.1 ± 1.2	39.8 ± 2.5	36.9 ± 1.7
69		16.7 ± 1.2	13.9 ± 1.9	32.3 ± 2.5	37.1 ± 1.8
76		27.8 ± 1.3	12.6 ± 1.1	29.4 ± 1.6	30.2 ± 1.6
83		18.3 ± 1.6	16.1 ± 2.1	31.3 ± 1.7	34.3 ± 0.8
90		18.7 ± 1.1	13.7 ± 1.1	36.2 ± 1.6	31.4 ± 1.1
27	Seeds	6.8 ± 0.9	4.1 ± 1.0	48.3 ± 1.6	40.8 ± 3.2
40		6.9 ± 0.6	4.1 ± 0.4	40.1 ± 2.3	48.9 ± 2.2
53		4.4 ± 0.7	4.4 ± 1.7	40.0 ± 1.9	51.2 ± 2.0
62		4.1 ± 0.5	4.2 ± 0.7	40.0 ± 2.0	51.7 ± 1.8
69		3.4 ± 0.8	4.3 ± 0.3	40.4 ± 2.6	51.9 ± 1.6
76		2.5 ± 0.5	3.8 ± 0.6	39.9 ± 2.6	53.8 ± 2.9
83		9.1 ± 0.3	9.2 ± 1.9	33.6 ± 1.6	48.1 ± 2.9
90		14.2 ± 1.5	10.6 ± 0.8	32.2 ± 1.3	43.0 ± 1.9

Values represent the mean ± s.e. of at least eight determinations.

C-6. Thus, the minute quantities observed confirm that our conditions were not detrimental to the sapogenins, although the recovery of these substances after hydrolysis of the saponins was total.

The dynamics of sterol and steroidal sapogenin accumulation led us to raise the question of the origin of these metabolites in fenugreek seeds. Clearly, for these compounds to be present in the dried seed, they had to be translocated to it or biosynthesized *in situ* during development. Translocation of sitosterol, mainly in the free form, has been observed in geranium and sunflower plants [28]. Transport of labelled cholesterol from the leaves to the fruits has also been shown in *Solanum khasianum* [29]. Sterols could also be translocated to maturing seeds of sorghum [30]. A possible translocation of diosgenin to the seeds has been reported in *Costus speciosus* [4]. Labelling experiments with [^{14}C]-mevalonate indicated the possibility that diosgenin is biosynthesized in leaves, then translocated in free form to the rhizome, flowers and seeds before being glycosylated and stored as the saponin form [5]. As previously reported, our results are consistent with the possibility of translocation from the vegetative parts to the pods, although no form of transport has been found. Conversely, biosynthesis of sterols has been shown in developing pea seed [6]. This capacity is greatest in the early stages and decreases during the development of the seed. But at each stage, it is more important than that observed in seedlings. The accumulation of sterols in the embryo and endosperm of developing maize kernels is considered to result uniquely from an *in situ* biosynthesis [14] but no biosynthesis was found in germinating seeds [18]. Steryl glycosides were synthesized using an enzyme preparation from immature soybean seeds [31] before a glucosyltransferase was found in pea seeds [7]. Biosynthesis of steroidal sapogenins in seeds or fruits has never been reported, mainly due to the fact that most of the species studied accumulate these compounds in their tuber or rhizome. We decided to investigate the possibility of such a biosynthesis in immature seeds of fenugreek pods because: (i) these seeds contain a higher level of cholesterol than that found in most of plants [9]; (ii) this sterol can be biosynthesized in seeds [6, 14] and is considered the main precursor in the biosynthesis of steroidal sapogenins [1, 8, 32]; (iii) our results show a good correlation between the changes in cholesterol level (Fig. 3) and the accumulation of steroidal sapogenins (Fig. 2B and D) during development and maturation. [2-^{14}C]-Acetate was administered to detached pods harvested at approximately 60 DAA. In a preliminary time-course experiment (see Experimental), the maximum specific radioactivity of the seeds (2.2×10^3 dpm mg^{-1} C) was measured after 24 hr uptake; detached pods were subsequently incubated for 24 hr. Free sterols, steryl conjugates, mono- and dihydroxylated sapogenins were then isolated from the seeds and purified. All these fractions were labelled and their radioactivity is given in Table 2. The ratio of incorporation into steryl esters, free sterols, acylsteryl glycosides and steryl glycosides was, respectively, 0.64:1:0.55:0.42. The ratio of labelling of total sapogenins to total sterols was 2.53. In order to verify the identity of labelled sapogenins, labelled diosgenin was isolated. A fraction containing 85% of diosgenin with a specific radioactivity of

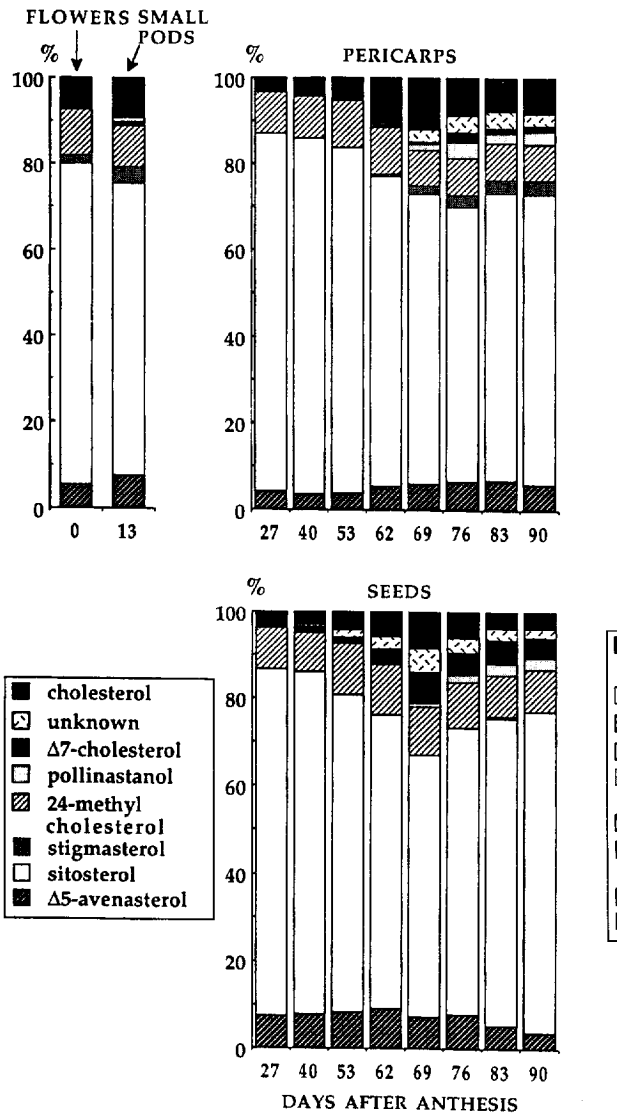


Fig. 3. Changes in the composition of sterols during pod development. The sterols taken into consideration account for at least 98% of total sterols. Results represent the mean of three to six determinations.

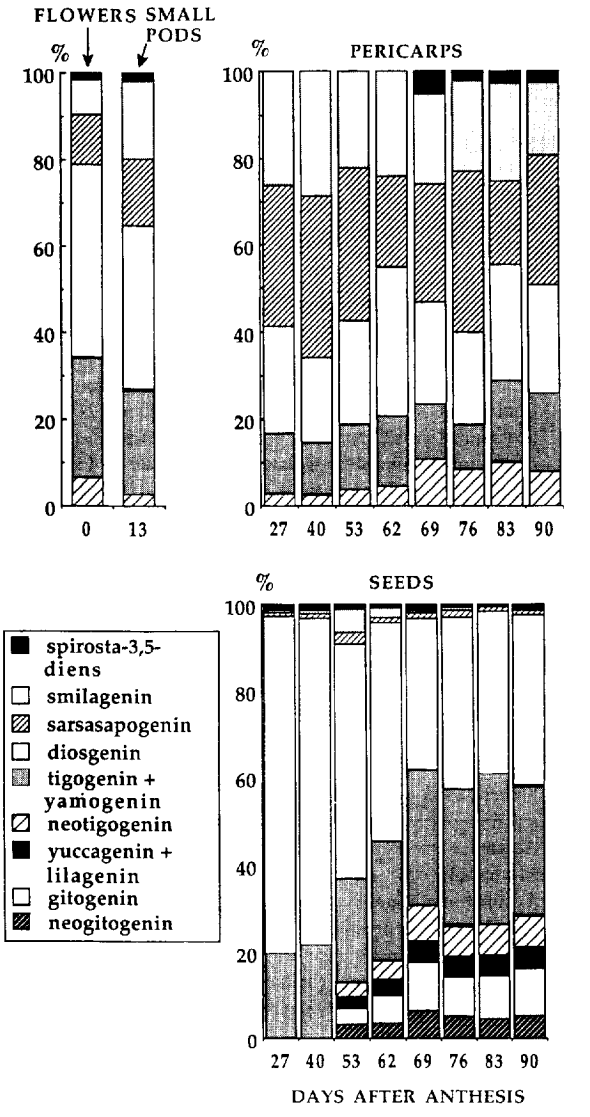
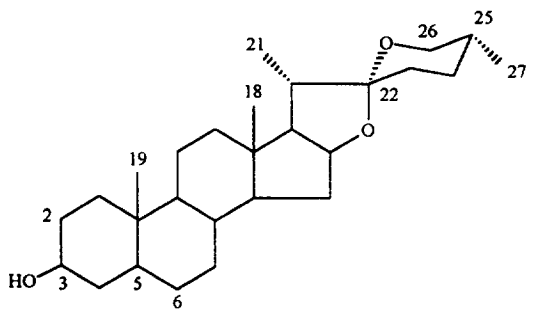


Fig. 4. Changes in the composition of steroidal sapogenins during pod development. Results represent the mean of three to six determinations.



401.9 dpm μmol^{-1} was obtained after purifications by TLC and HPLC. Pure carrier diosgenin was added and this fraction was crystallized to constant specific radioactivity (Table 3). Finally, our results indicate that excised pods of fenugreek can biosynthesize diosgenin and other steroids.

The glycosylations involved in the biosynthesis of steroidal saponins from the corresponding sapogenins have been widely investigated in the few past years [33,34]. However, there are very few data on the biosynthesis pathway of steroidal sapogenins. Cholesterol is considered the main precursor in the biosynthesis of these compounds [1,32] but the question arises whether the next step is a glycosylation at C_3 [8,35] or an hydroxylation at C_{26} or C_{27} [29,36]; another hydroxylation at C_{22} could be involved in the last reactions [37]. Fenugreek pods and seeds could provide useful experimental matter to investigate other steps in this pathway and to contribute to the knowledge of the origin and function of steroidal sapogenins or saponins in plants.

Table 2. Distribution of radioactivity in the seeds of one pod following feeding of excised pods with [2-^{14}C]-acetate

Fractions	Total radioactivity (dpm)
Steryl esters	273 ± 27
Free sterols	426 ± 86
Acylsteryl glycosides	235 ± 12
Steryl glycosides	180 ± 51
Monohydroxylated sapogenins	2508 ± 318
Dihydroxylated sapogenins	310 ± 39

30 pods were incubated individually in a solution containing 500 μl Na_2EDTA 20 mM and 200 μl [2-^{14}C]-acetate (2.18×10^6 dpm per pod). Sterolic forms and steroidal sapogenins were isolated as described in Experimental. The radioactivity of the hexane and iso-PrOH extracts was, respectively, 3.6×10^3 and 29.0×10^3 dpm (seeds of one pod)$^{-1}$. The values represent the mean ± s.e. of three measures of different aliquots.

Table 3. Purification of labelled diosgenin to constant specific radioactivity

Solvent used for crystallization	Specific radioactivity (dpm μmol^{-1})
Acetone	121 ± 6
Methanol	125 ± 10
Acetone–methanol (1:1)	122 ± 3
Hexane–dichloromethane (1:1)	126 ± 3

The values represent the mean ± s.e. of three quantifications and radioactivity measurements of the diosgenin.

EXPERIMENTAL

Plant material. The fenugreek plants (*T. foenum-graecum* cv. ghouka) for the study of development and maturation were grown in our experimental field. Seeds were sown in October. Flowers ($n = 900$) or pods ($n = 300$) were harvested at various days after anthesis in June and July. Experiments were run for 2 years. Flowers or pericarps and seeds from the pods were freeze-dried for 48 hr. Plants for labelling experiments were grown throughout the year in a greenhouse. Pods were harvested at approximately 60 DAA. Pericarps and seeds were separated and freeze-dried as above.

Extraction of sterols and steroidal sapogenins. Sterols and steroidal sapogenins were extracted according to a modified method [38]. Powdered samples (10 g) were extracted in a Soxhlet apparatus with hexane for 12 hr followed by iso-PrOH–H_2O (7:3) for 18 hr. Each extract was taken to dryness under reduced pressure and redissolved in 10 ml of hexane or iso-PrOH–H_2O (7:3) respectively.

Analysis of total sterols. Int. standard (1 ml) betulinol [39] (0.5 mg ml^{-1} 100% EtOH) was added to 1 ml of both hexane and iso-PrOH–H_2O extracts and evapd to dryness under reduced pressure. The residue was redissolved in 0.09 M H_2SO_4 in 95% EtOH (8 ml) and refluxed for 12 hr to cleave the steryl glycosides. KOH (3 ml, 1.8 M, in 95% EtOH) was added and refluxed for 1 h to hydrolyse steryl esters. These conditions were adapted from [40]. Total sterols were extracted from the above solution with Et_2O (20 ml × 3) after addition of H_2O (11 ml). The three fractions were combined, washed with H_2O (20 ml × 3) and taken to dryness under reduced pressure. The residue was redissolved in $CHCl_3$ (500 μl). Total demethyl sterols were purified by TLC on silica gel with $CHCl_3$–Et_2O (9:1) as developing solvent. Cholesterol was used as a standard for the identification of the sterol band (R_f 0.37) and a spray soln of berberine (0.1% in 95% EtOH) for detection at 366 nm. The corresponding zone was scraped off and extracted with $CHCl_3$–MeOH (2:1). The residue was taken to dryness under N_2. Sterols were acetylated overnight at room temp. with 450 μl of acetic anhydride–pyridine (2:1). Samples were evapd to dryness under N_2 and resuspended in an appropriate volume of EtOAc prior to GC analysis. The capillary column used was a WCOT (30 m, 0.25 mm i.d.) coated with DB-1 (0.25 μm). Temp. 280° isothermal for the column and 300° for inlet and detector (FID). H_2 was used as carrier gas at a pressure of 0.85 bar, split 73 ml min^{-1}.

Identity of sterols. Verified by GC-MS. The column and temperature conditions were the same as for GC. A more polar column (WCOT, 25 m, 0.25 mm i.d., 0.1 μm OV-17) was also used to confirm the identifications. Temp. conditions: 250° for oven and 280° for inlet and detector. He was used as carrier gas (0.5 bar), ion source 150° and ionizing energy 70 eV.

Determination of the proportions of sterol conjugates. Aliquots of 100 μl of both hexane and iso-PrOH–H_2O extracts were combined in a microtube and evapd. to dryness under N_2. Residue was redissolved in 100 μl of $CHCl_3$–MeOH (2:1). A standard of cholesterol (free sterol, FS) was used for quantification. Standards of sterylesters SE (from commercial source), steryl glycosides (SG) and acylsteryl glycosides (ASG) (purified in our laboratory) were used for the identification of the other sterolic forms. The four sterolic forms were separated by TLC on silica gel (20 × 20 cm) using two successive developments with A, hexane–EtOAc (92:8, run of 18 cm), and B, CH_2Cl_2–MeOH–H_2O (90:10:0.5, run of 12 cm). The plate was dried at room temp. after each development. The four sterolic forms were identified after spraying the plate with a 9 M H_2SO_4 soln in 100% MeOH and heating for 10 min at 110°. Under these conditions R_f values were 0.11 for SG, 0.23 for ASG, 0.40 for FS and 0.64 for SE. The proportions of the sterolic forms were determined by photodensitometry with a Vernon PHI 6 instrument.

Analysis of total steroidal sapogenins. According to the maturation stage, 1–4 ml of the internal standard solution of betulinol (0.5 mg ml^{-1} 100% EtOH) were added to 1 ml of the iso-PrOH–H_2O extract (steroidal saponins are present only in this extract) and taken to dryness

under reduced pressure. This fraction was hydrolysed with 15 mL of 1 M H_2SO_4 in iso-PrOH–H_2O (7:3) and refluxed for 12 h [41]. This reaction cleaved the sapogenin-sugars bonds of saponins. The amount of alcohol was reduced to 30% by addition of H_2O. Total steroidal sapogenins were extracted from the above soln with Et_2O (20 ml × 3) and the three fractions were combined. This soln was made alkaline with 0.9 M KOH aq. (20 ml), washed with H_2O (20 ml × 3) and evapd to dryness under reduced pressure. Acetylation and GC quantifications were performed under the same conditions as for the total sterols. Identifications were also verified by GC-MS (equipped with the DB-1 column).

General labelling procedure. Excised pods were individually incubated by the pedicel in a soln containing 500 μl Na_2EDTA 20 mM [42] and 200 μl [2-^{14}C]-acetate as the sodium salt (sp. act. 2.0 GBq mmol^{-1}). The pods were kept in this soln under a light irradiation of 86 μmol m^{-2} s^{-1} from HPIT 400 W lamps at 20° for the time of incubation desired. Pericarps and seeds were then separated and freeze-dried as above.

Determination of the uptake of [2-^{14}*C*]*-acetate.* Each pod was supplied with 2.09×10^6 dpm [2-^{14}C]-acetate and incubated for 1, 2, 3, 5, 9, 24 and 48 hr (10 pods each). Seeds were isolated and freeze-dried. After grinding and dry combustion (900°) of the samples in a stream of O_2, a dosing pump sepd the resulting gases into three different frs [42]. Gas, 93.2%, passed through a soln of methyl cellosolve–monoethanolamine (3:1) absorbing $^{14}CO_2$ and radioactivity was counted using appropriate scintillation liquid; 1.6% diverted towards the total C titration unit. CO_2 was absorbed in a soln of NaOH and total C was automatically determined by a carbon analyser; 5.2% was discarded through a safety trap. The specific radioactivity was expressed as dpm (from $^{14}CO_2$) mg^{-1} total C.

Labelling of sterols and steroidal sapogenins. Each pod ($n = 30$) was supplied with 2.18×10^6 dpm [2-^{14}C]-acetate. Sterols and steroidal sapogenins from the seeds were extracted as above. The four sterolic forms were purified once by TLC on silica gel (0.25 mm analytical plates used as preparatives) with the solvents A and B and identified with R_f values and standard substances detected as above. Sterolic forms were recovered from the gel with $CHCl_3$–MeOH (2:1). These frs were repurified using only solvent A for FS, solvent B for SG and ASG (run of 18 cm) or hexane–Et_2O (94:6) for SE. Mono- and dihydroxylated sapogenins were isolated twice by TLC on silica gel using hexane–EtOAc (6:4) and recovered with iso-PrOH–H_2O (7:3). Aliquots of these fractions were taken for radioactivity measurement using appropriate scintillation liquid.

Isolation of labelled diosgenin. The diosgenin was isolated from the fraction of labelled monohydroxylated sapogenins by HPLC using UV detection at 210 nm. A binary solvent gradient (H_2O–acetonitrile) was used: 40:60 for 2 min, adjusted to 0:100 at 14 min and held for 20 min. Samples (20 μl), were injected on a C18 Kromasil column (250 × 4 mm i.d.) and solvent was pumped at a rate of 1.5 ml min^{-1}. Frs collected were combined and evapd under N_2. An aliquot was taken for radioactivity measurement. After addition of pure nonlabelled diosgenin as carrier material, the diosgenin was crystallized to constant specific radioactivity and the results are given in Table 3.

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