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Characterization of Radish (*Raphanus sativus*) Storage Proteins[1]

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ABSTRACT

Radish (*Raphanus sativus* cv Rond rose à bout blanc Vilmorin) seeds, as other cruciferae oil seeds, contain two major types of storage protein aggregates which can be separated by gel filtration into 12 and 1.7 Svedberg fractions. These two fractions have been characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, amino acid composition, and two bidimensional gel electrophoresis systems. These results were compared with those obtained with rapeseed storage proteins. Radish 12 Svedberg particles are made of a series of nine major polypeptides ranging from 33 to 30 kilodaltons. These polypeptides present charge heterogeneity. The 12 Svedberg particle is made of six subunits ≃ 55 kilodaltons. Each subunit is a couple of two polypeptides linked by a disulfide bridge. The 1.7 Svedberg particle has a simpler composition. It is made of two polypeptides of 10 and 12 kilodaltons and smaller peptides of ≃ 7 kilodaltons. Twelve and 1.7 Svedberg particles also differ in their amino acid composition, the 1.7 Svedberg being particularly rich in glutamic acid and proline. Its components are basic. The organization of the rapeseed storage protein is similar but more complex.

Storage proteins constitute the principal protein component of the seed. The genes which code for them are developmentally regulated and are expressed only during seed formation and maturation stages. We have been studying radish seed formation and germination at a molecular level during recent years (1, 2, 8–10). As a first step towards the isolation of genes coding for abundant seed proteins, we have purified and partially characterized the major storage proteins from this oil seed species. There is no report, to our knowledge, dealing with this protein family in radish. Curiously, even in closely related, economically important species, such as *Brassica* and *Sinapis*, storage proteins have been very poorly characterized, so that little information is available about their complexity and structure when compared to the extensively studied legume or cereal storage proteins (4, 28). *Brassica*, *Sinapis*, and radish storage proteins also accumulate in protein bodies (12, 17; M. Laroche *et al.*, unpublished). Two classes of storage proteins recognized in *Brassica* and *Sinapis* have been named 12 and 1.7 S proteins according to their S value (3, 11, 14, 15, 19, 21). Most often their characterization has been limited to nondenaturing one-dimensional gel electrophoresis, amino acid composition, and gel filtration. Only recently have polyacrylamide gel electrophoresis experiments been carried out in denaturing conditions. They have revealed that *Brassica napus* 12 S protein is made of four polypeptides whereas 1.7 S is made of two (5, 27).

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In this paper, we characterize radish 12 and 1.7 S proteins and compare them to their rapeseed equivalents. SDS-polyacrylamide gel patterns and amino acid compositions are reported. In addition we have analyzed each family using two different two-dimensional gel electrophoresis systems. Combining isoelectrofocusing in the first dimension and SDS in the second, we demonstrated that the 12 S family is much more complex than previously assumed. Finally, using a second system in which the first dimension is run in nonreducing conditions whereas the second is in the presence of a reducing agent, we obtained information concerning the associations between the different polypeptides.

MATERIALS AND METHODS

Extraction and Purification of Storage Proteins. Radish seeds (cv Rond rose à bout blanc, Vilmorin) and rapeseeds (cv Jet Neuf) were ground in liquid N_2 and extracted as described previously (5). The extract was fractionated into aliquots which were stored frozen at −80°C until use. The 12 and 1.7 S storage protein particles were purified by gel filtration through a Sepharose 6B column (90 × 1 cm) equilibrated in 10 mM sodium phosphate, 0.5 M NaCl, pH 7.5. Fractions were dialyzed against distilled H_2O, and freeze-dried. Each fraction was then analyzed on a SDS-polyacrylamide gel and those containing the storage proteins were identified. In subsequent preparations, the elution pattern turned out to be very reproducible and the fractions containing the storage proteins were immediately pooled, dialyzed, and freeze-dried. The protein content in each fraction was determined using the Lowry method (20).

Polyacrylamide Gel Electrophoresis. To separate proteins in one dimension, the SDS-polyacrylamide gel system (18) was employed, using either 12.5 or 17% slab gels. Gels were stained with amido black or Coomassie blue R-250. Amido black-stained gels were routinely destained and further processed with the silver nitrate staining procedure (24). The isoelectrofocusing cylindrical gels and the second dimension were run as previously described (23, 25) using a pH range 3.5 to 10. The second two-dimensional gel system was essentially as already published (22). The first dimension was run on a 1-mm thick slab containing 12.5% acrylamide gel in the absence of 2-mercaptoethanol. Each sample was run in duplicate. One strip was stained with Coomassie blue and the other was incubated in sample buffer containing 5% 2-mercaptoethanol to reduce the protein subunits and was placed on the stacking gel of a second dimension 1.5 mm thick, 17% acrylamide. This second dimension gel was stained with Coomassie blue. Molecular weight standards were included in each SDS-gel electrophoresis separation.

Amino Acid Composition. Amino acid composition was determined with an automatic Beckman amino acid analyzer. Samples were hydrolyzed *in vacuo* for 36 h in the presence of 6 N HCl at 110°C.

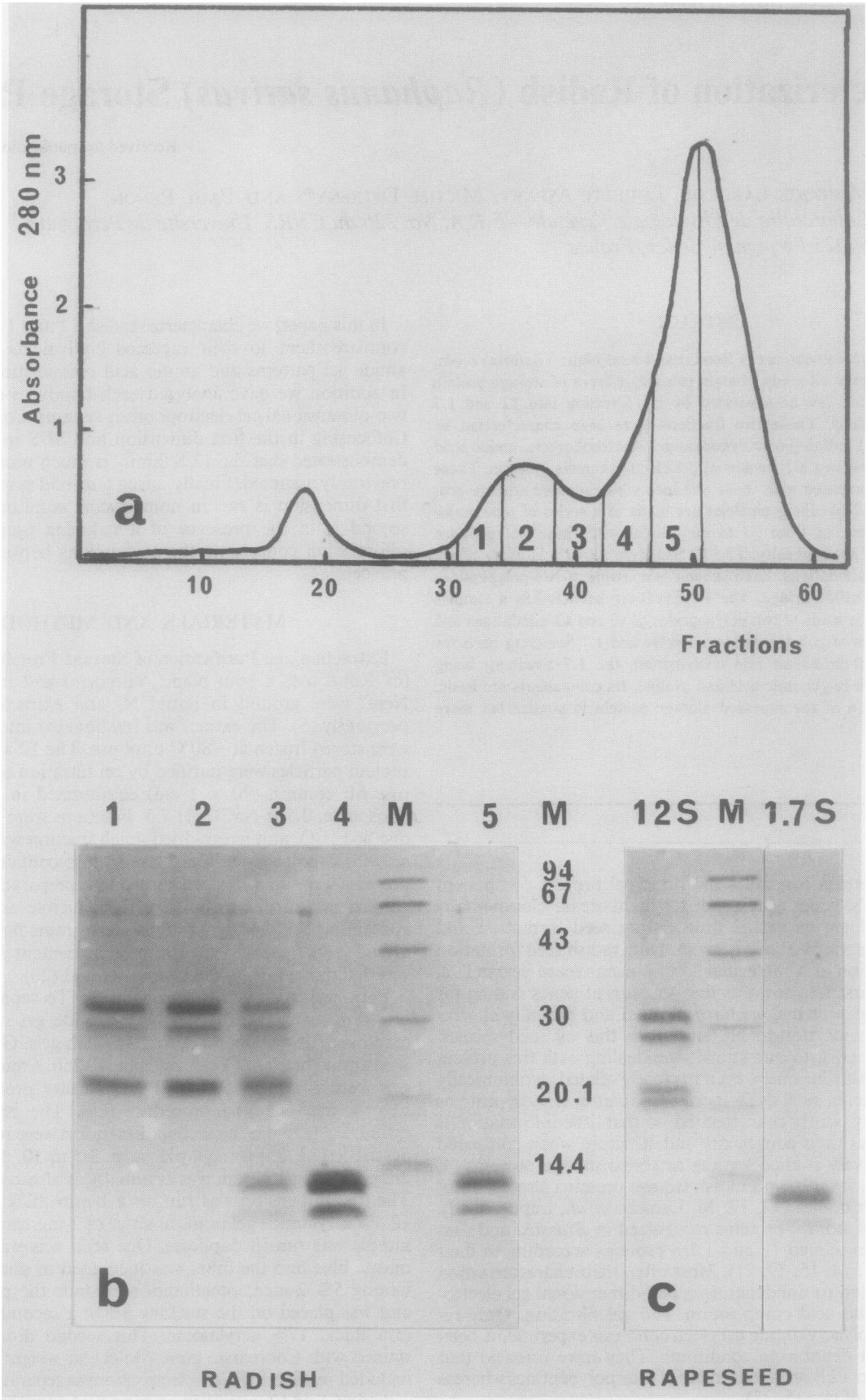

FIG. 1. Purification of radish storage proteins. Gel filtration was carried out on a Sepharose 6B column at 4°C equilibrated with 10 mM sodium phosphate buffer, pH 7.5, 0.5 M NaCl, and 1 mM phenylmethylsulfonyl fluoride, a protease inhibitor. Four-ml fractions were collected, pooled as indicated on the elution pattern (a), and analyzed on a 12.5% SDS-polyacrylamide gel (b). Slot numbers correspond to the pooled fractions numbers. Twenty μg were loaded in each slot. Rapeseed 12 and 1.7 S proteins purified using the same procedure are shown in (c) for comparison. Molecular weight markers are phosphorylase *b* (94 kD), BSA (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20.1 kD), and α-lactalbumin (14.4 kD). The gels were stained with Coomassie blue.

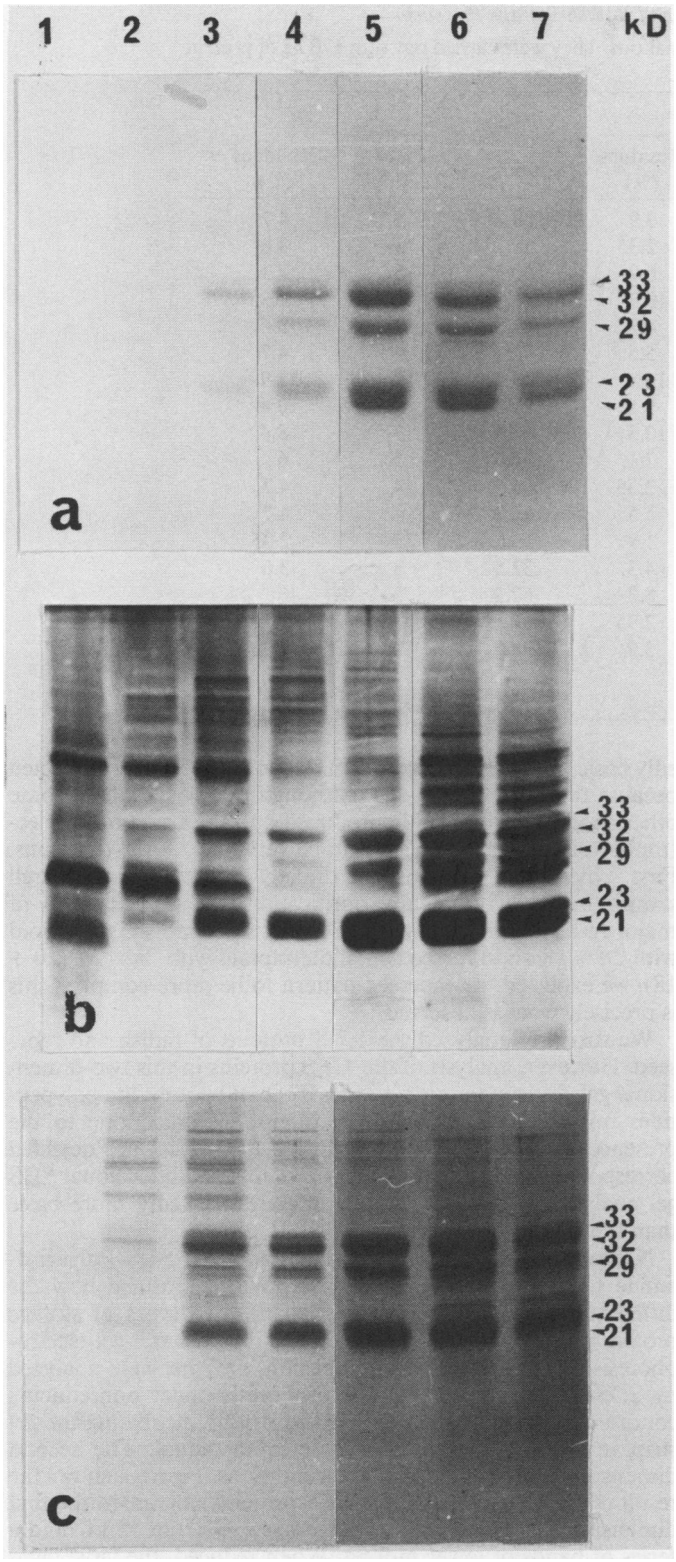

FIG. 2. Analysis of a Sepharose 6B column eluate using different staining procedures. Pooled fractions 15 to 16 (lane 1), 20 to 22 (lane 2), 28 to 30 (lane 3), 31 to 32 (lane 4), 33 to 34 (lane 5), 35 to 36 (lane 6), and 37 to 38 (lane 7) eluted from a Sepharose 6B column loaded with a radish extract were run on two SDS-12.5% polyacrylamide gels. The protein amounts loaded on the gels were 10, 10, 16, 60, 100, 93, and 58 μg in slots 1 to 7, respectively. The protein amount in fractions 15 to 16 (lane 1) and 20 to 22 (lane 2) is probably overestimated in the Lowry assay due to interference of nucleic acids which are the main component of these fractions. (a) and (b) show the first gel stained with amido black (a) or destained after photography and restained using the silver nitrate procedure (b). (c) shows the second gel stained with Coomassie blue. Molecular weights of the major 12 S polypeptides are indicated.

RESULTS

Purification and Identification of Radish Storage Proteins. The major radish storage proteins were identified by comparison with those of rapeseed using the same method of preparation. Figure 1 shows the elution pattern of a radish crude extract after passage through a Sepharose 6B column. The SDS-polyacrylamide gel electrophoresis pattern (18) of representative fractions is shown and is compared with similar fractions from rapeseed. The rapeseed patterns of 12 and 1.7 S fractions are very characteristic and are virtually identical to those published by other groups (5, 27). As shown in Figure 1, corresponding fractions can be purified from a crude extract of radish. Storage proteins account for most of the proteins in the dry radish seeds. Radish and rapeseed 12 and 1.7 S proteins have very similar SDS-polyacrylamide gel patterns although there are slight differences in the mol wt of the different polypeptides. This suggests a close relationship between the polypeptide components of storage proteins in the two species.

The mol wt of the major radish storage protein bands have been estimated to be 33, 32, 29, and 23 to 21 kD for the 12 S fraction. A double band at 10 to 12 kD and a band corresponding to a smaller mol wt protein of ≃7 kD are detected on the 1.7 S pattern. All these bands are rather large and it is very probable that each one corresponds to several slightly different polypeptides. This assumption was confirmed by two-dimensional gel electrophoresis as shown below.

To estimate the purity of the 12 and 1.7 S fractions, different staining procedures were used (Fig. 2). Staining the gel with amido black (Fig. 2a) suggests that the 12 S fraction has been purified to homogeneity. After Coomassie blue staining (Fig. 2c), the purity of the 12 S fraction still seems quite reasonable, more than 80% of the stained material being in the storage protein bands. On the other hand, silver staining reveals a considerable amount of other material (Fig. 2b). However, it is clear that, although very sensitive, this staining does not give reliable quantitative information. For instance, the 23 to 21 kD bands are always more heavily stained than the 33 to 32 kD bands. Another example of differential staining is observed in the first fractions eluted from the column. As estimated from gels stained with amido black or Coomassie blue and from Lowry determinations, these fractions contain very little protein, yet several bands are stained as intensely as storage proteins after silver staining. Another difficulty in estimating protein amounts after silver staining is that not all proteins stain the same way. Some stain yellow or orange (33, 32, and 29 kD) and others stain dark brown (23–21 kD). Similar results (not shown) were obtained when analyzing the 1.7 S fraction.

We conclude from this one-dimensional gel analysis that the two protein fractions that we have isolated were reasonably pure and we characterized them further by determining their amino acid composition.

Amino Acid Composition of the 12 and 1.7 S Radish Storage Proteins. The amino acid composition of the two fractions is presented in Table I. We have assumed an average mol wt of 30 kD for the 12 S and 10 kD for the 1.7 S fraction in order to calculate an average residue number. These values are only approximate, since each fraction is made of several subunits with different mol wt. However, the values for the 1.7 S proteins correspond closely to those published for rapeseed (19) and to the composition derived by cDNA sequencing (6, 7). The 12 S fraction is very rich in aspartic and glutamic acids, these two amino acids representing more than 25% of the residues. Since

Table I. *Amino Acid Composition of Radish Storage Proteins*

The values are the average of two independent determinations. They were carried out with 170 μg of protein for 12 S and 130 μg for 1.7 S.

Amino Acid	12 S			1.7 S		
	nmol	No. of Residues	Residues (%)	nmol	No. of Residues	Residues (%)
Lysine	56.7	10	3.9	50.0	4	4.7
Histidine	33.9	6	2.35	36.7	3	3.6
Arginine	84.6	15	6.0	81.5	6	7.2
Aspartic acid	145.7	26	10.3	26.9	2	2.35
Threonine	70.7	12	4.7	42.8	3	3.6
Serine	80.5	14	5.5	48.7	4	4.7
Glutamic acid	228.2	40	16.3	243.3	19	22.9
Proline	79.4	14	5.5	121.7	9	10.8
Glycine	147.4	26	10.3	86.0	7	8.4
Alanine	103.3	18	7.1	67.6	5	6
Half-cystine	11.3	6	2.35	48.6	4	4.7
Valine	81.7	14	5.5	52.2	4	4.7
Methionine	25.5	5	1.9	19.6	2	2.35
Isoleucine	63.7	11	4.3	32.5	3	3.6
Leucine	121.3	21	8.3	67.3	5	6
Tyrosine	33.4	6	2.35	17.1	1	1.2
Phenylalanine	57.6	10	3.9	28.0	2	2.35
Total		251			83	

the 12 S protein is rather neutral, as shown below, many of the acidic residues should be present in the proteins as amides. There are some striking differences between the amino acid compositions of the two fractions. Although the 1.7 S fraction is also very rich in glutamic acid, it contains much less aspartic acid. However, the sum of the acidic residues is still about 25%. The 1.7 S fraction is rather proline-rich. Similar differences between the two fractions have been noticed in various *Brassica* (12, 19).

Isoelectrofocusing—SDS Two-Dimensional Polyacrylamide Gel Electrophoresis Analysis. The two-dimensional isoelectrofocusing—SDS-polyacrylamide gel pattern of radish 12 S storage protein fraction is shown in Figure 3a. It clearly demonstrates that each band from the one-dimensional SDS gel can be resolved into a series of spots corresponding to polypeptides with similar mol wt but with different charges. At least nine major spots (lettered A to I) can be distinguished, indicating that the composition of the 12 S aggregate is much more complex than indicated by monodimensional SDS-polyacrylamide gel electrophoresis. We mentioned that the one-dimensional pattern of radish was very close to that of rapeseed. Since rapeseed two-dimensional isoelectrofocusing SDS-polyacrylamide gel electrophoresis has not yet been reported, we also ran a 12 S rapeseed sample under identical conditions (Fig. 3b). The two patterns are clearly different and the rapeseed 12 S protein pattern is more complex than that of the corresponding radish fraction. For instance, in rapeseed there are four major spots and several minor ones in place of A and B. Spot C is replaced by a series of four major and several minor spots. In place of the four spots D, E, F, and G there are six different spots. On the other hand, spots H and I are apparently missing in the rapeseed extracts. In addition, a new series of spots corresponding to polypeptides of ≃50 to 55 kD show up on the rapeseed pattern. However, these polypeptides are present in variable amounts from one experiment to another and we suspect that these spots arise from aggregates which are not fully reduced when the gels are overloaded.

All the spots are located in the center of the gel which indicates that the 12 S proteins are neutral. However, it seems that rapeseed contains a less abundant family of polypeptides which are markedly basic (indicated by arrows). This family is probably detected because the gel is overloaded. Although it is possible that some other spots are artifactually produced during extraction or electrophoresis, this does not seem to be very likely for two reasons. First, when total proteins are directly extracted in O'Farrell sample buffer, without any purification, the same pattern of major spots appears on the gel. Second, since radish is a diploid with $2n = 18$ and rapeseed an allotetraploid with $2n = 38$ (20 + 18), we expected the rapeseed pattern to be more complex: this is precisely what is observed.

We similarly analyzed the 1.7 S proteins of radish and rapeseed. However, analysis of the 1.7 S proteins in this two-dimensional gel electrophoresis system is difficult because the polypeptides migrate in a region more intensely stained due to the presence of ampholines. Nevertheless, two spots are detected corresponding to the bands identified in one-dimensional SDS gel analysis. The 1.7 S components are markedly more basic than their 12 S counterparts.

Nonreducing-Reducing Two-Dimensional SDS-Polyacrylamide Gel Electrophoresis Analysis. We determined how the different peptides were associated in the two types of storage protein particles by using another two-dimensional gel electrophoresis system. In the first dimension, proteins were analyzed by SDS-polyacrylamide gel electrophoresis under nonreducing conditions. They are then reduced *in situ* by incubating the gel strip in the presence of 5% 2-mercaptoethanol. The second dimension is run under reducing conditions. Figure 4 shows the result of such an analysis. Twelve S proteins migrate in the first dimension as a large band with average mol wt of 55 kD and a series of bands of lower mol wt. When reduced, the large band of ≃55 kD gives rise to a series of spots aligned along a vertical line with respective mol wt of 33, 32, 29, and 23 to 21 kD. It is therefore reasonable to assume that each peptide in the range of 30 kD is associated by a disulfide bond with a peptide in the range of 20 kD to give a dimer of ≃55 kD. Since the mol wt of the 12 S particle is about 300 to 350 kD, as estimated by gel filtration, there should be six 55 kD dimers associated by hydrogen bonds in a 12 S particle. It should be noticed that among the other minor bands detected in the first dimension three (33, 31,

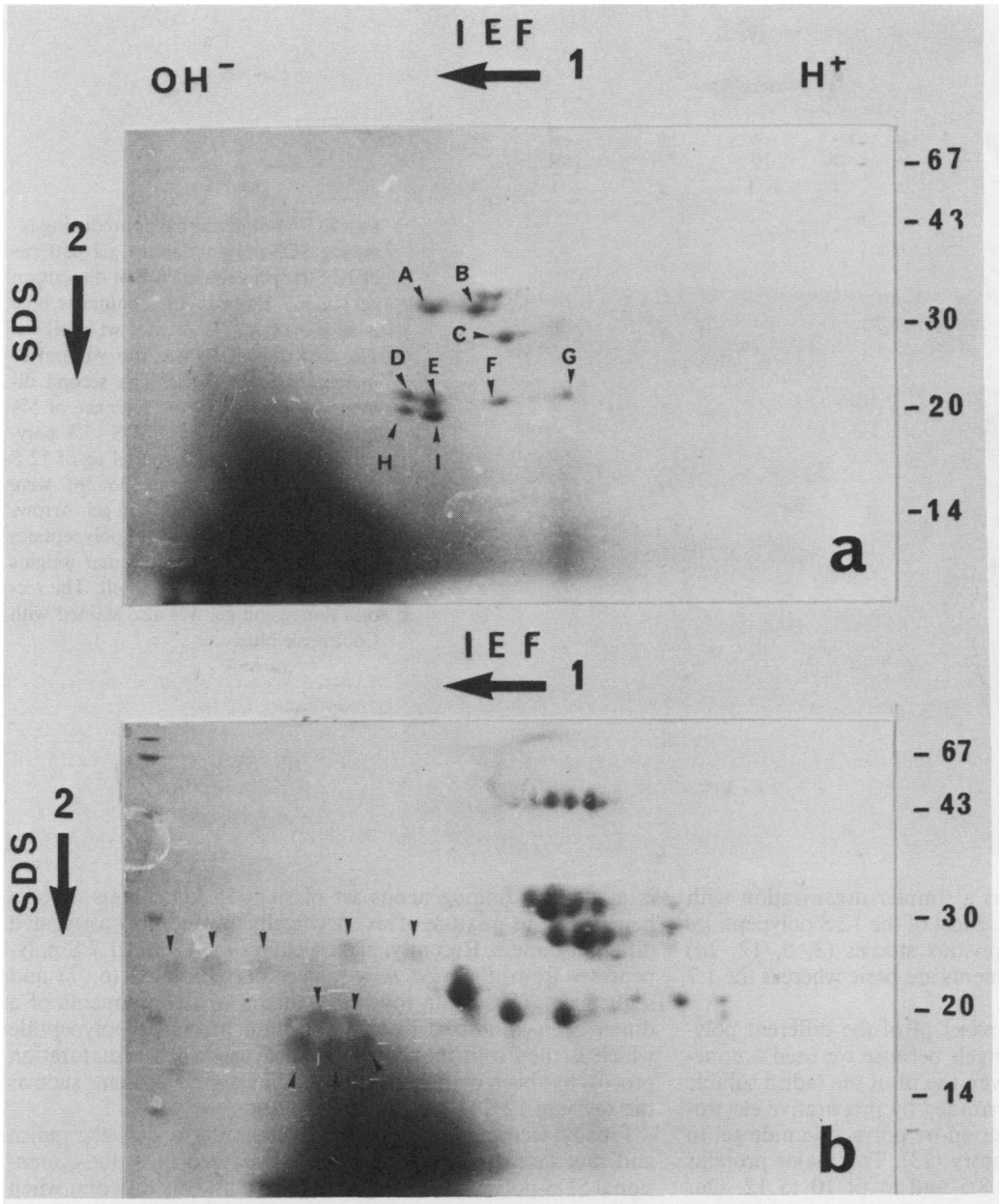

FIG. 3. Two-dimensional isoelectrofocusing-SDS-polyacrylamide gels of radish (a) and rapeseed (b) 12 S protein. Seventy μg of radish and 160 μg of rapeseed 12 S proteins were loaded on the isoelectrofocusing (IEF) gel. Fractions 35 to 36 were used for this experiment. Both gels were stained with amido black. Major protein spots are lettered A to I in order to help comparison. Molecular weights are indicated on the left. Arrows on the rapeseed gel point to minor families of basic polypeptides. First and second dimensions are as indicated.

and 30 kD, indicated by arrows) dissociate into two series of spots with respective mol wt of 21 and 14 kD, 13 and 10 kD under reducing conditions. This family of polypeptides does not correspond to contaminating 1.7 S particles since, as shown below, they have a different pattern. It is possible that it is a minor storage protein family of intermediate size which was detected due to overloading of the gels. We cannot rule out that these small polypeptides arise by specific cleavage of the major proteins of mol wt 33, 32, and 29 kD. However, a similar set of polypeptides is detected when the rapeseed 12 S protein is analyzed using this method (not shown). These polypeptides have mol wt which correspond to those of the minor family of basic polypeptides detected by two-dimensional isoelectrofocusing—SDS-polyacrylamide gel electrophoresis. We therefore infer that radish 12 S proteins also contain a minor family of polypeptides in addition to the principal components. Finally, it should be noticed that there are a series of spots migrating along a diagonal. This suggests that these polypeptides were present in the 12 S particles but they are not linked with other components by disulfide bonds. Whether these polypeptides are identical to those present in the 55 kD dimers or different remains to be established.

The 1.7 S fraction was analyzed using this same procedure. These proteins migrate as a large band in the first dimension. They dissociate upon reduction into smaller fragments of about 12 and 11 kD polypeptides and diffuse spots of smaller size. The 12 and 11 kD are not aligned on the same vertical line, suggesting that the 1.7 S fraction is made of two distinct aggregates of slightly different size (Fig. 5).

DISCUSSION

In this paper, we report on the characterization of the principal storage proteins from radish and, to a lesser extent, from rapeseed. SDS-polyacrylamide gel electrophoresis and amino acid composition studies confirm previous observations on rapeseed and other related species (3, 5, 11, 12, 14, 15, 19, 21, 26, 27). However, the use of two-dimensional gel electrophoresis systems provides new information.

The 12 S particle is made of a larger number of polypeptides than was assumed from one-dimensional gel analysis. On the

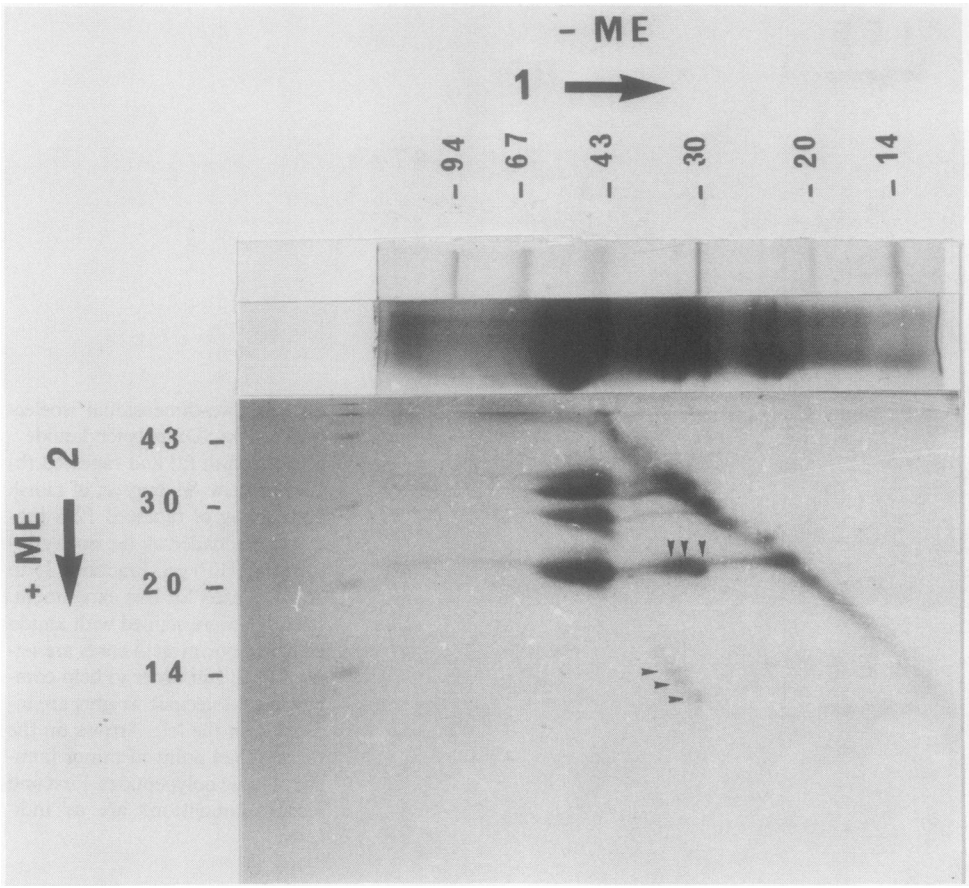

FIG. 4. Two-dimensional nonreducing-reducing SDS-polyacrylamide gel patterns of 12 S radish protein. A first dimension gel (12.5%) stained with Coomassie blue is shown on top with mol wt markers. The first dimension was run without 2-mercaptoethanol (ME). The second dimension was run in the presence of 5% 2-mercaptoethanol on a SDS-17% polyacrylamide gel. Two hundred μg of 12 S radish protein (fractions 35–36) were loaded on the first dimension gel. Arrows point to the minor family of polypeptides discussed in the text. Molecular weights in kD are indicated on the left. The second dimension gel was also stained with Coomassie blue.

other hand, the 1.7 S protein has a simpler organization with two major peptides ≃10 to 12 kD. Most of the 12 S polypeptides are neutral as determined in previous studies (3, 5, 12, 14) although a series of minor components are basic whereas the 1.7 S components are basic.

It is difficult to determine the exact pI of the different polypeptides in each family from our gels because we used a nonequilibrium technique (25). However, the pI of the radish soluble cotyledon proteins had been determined by preparative electrofocusing and analysis of each fraction by polyacrylamide gel in a previous study from our laboratory (13). The major proteins were found between pI 6.2 and 8.5 and at pI 10 to 12. Our observations are in good agreement with these data.

The heterogeneity of the 12 S fraction is striking, particularly that of rapeseed. Several factors may contribute to the heterogeneity revealed by isoelectrofocusing. First, there could be different polypeptides coded by different genes. Second, some of the spots may correspond to polypeptides modified by glycosylation or other post-translational modification. Third, the heterogeneity could arise during extraction or electrophoresis. In the light of results obtained with other plants (4, 28), the radish and rapeseed storage proteins are probably encoded by a multigene family. In addition, preliminary results indicate that 12 S radish storage protein is glycosylated and contains 2.6% glucose confirming a previous report on *Brassica napus* (14).

The structure of the 12 S particle of *Brassica napus* has been investigated recently using physical methods (26) and it has been suggested that it is made of six globular subunits. Our results give some support to this model if one considers that a subunit is a coupling of two polypeptides bonded by a disulfide bridge. However, due to the heterogeneous composition of the 12 S fraction, there is presumably not a single type of 12 S protein. At the moment, we do not know whether a given 12 S particle is made of a homogeneous set of six ≃55 kD dimers or of a heterogeneous mixture of six specifically or randomly associated different dimers. Recently, cDNA clones for 12 and 1.7 S polypeptides from *Brassica napus* have been isolated (6, 7) and sequenced. These data indicate that the two components of a dimer are synthesized from a common precursor polypeptide which is then split by proteolytic cleavage. Such a maturation process has been demonstrated for other storage proteins such as the soybean 12 S glycinin complex (29).

Finally, we note that despite a great similarity between radish and rapeseed storage proteins when analyzed in a one-dimensional SDS-polyacrylamide gel they are markedly different when analyzed on a two-dimensional isoelectrofocusing-SDS gel. Preliminary results indicate that antibodies raised against the radish 12 S protein recognize rapeseed 12 S component. A rapeseed cDNA clone corresponding to a 12 S component (kindly given by Dr Crouch) may be useful as a probe to specifically detect homologous fragments in the radish genome. Despite these close relationships, it is very easy to distinguish between radish and rapeseed proteins using a combination of isoelectrofocusing and SDS—denaturing polyacrylamide gel. This very powerful technique used in association with immunological and molecular techniques should be useful in analyzing the regulation of expression of this complex gene family and in screening the numerous available cultivars for structural or regulatory mutants. This should also shed a new light on taxonomic and evolutionary studies (30, 16) among *Brassica* and, more generally, cruciferae by providing reliable molecular markers.

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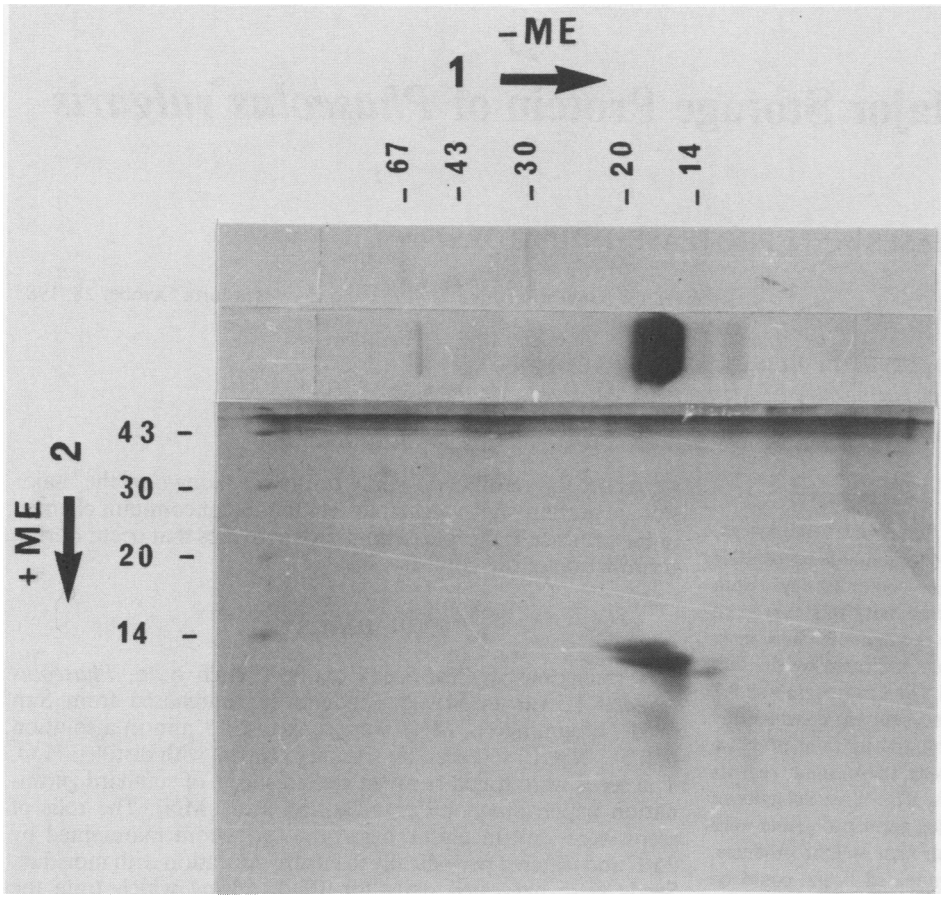

FIG. 5. Two-dimensional nonreducing-reducing SDS-polyacrylamide gel pattern of 1.7 S radish protein. The first and second dimensions were run as indicated in the legend to Figure 4 except that 100 μg of 1.7 S protein were loaded on the first dimension gel.

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