Cysteine Sulfoxides and Alliinase Activity of Some *Allium* Species

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The flavor precursors of 17 species belonging to the Alliaceae family were analyzed by HPLC, and results were evaluated with respect to the classification of species into their genus, subgenus, and section. Identification and quantification of these precursors were carried out by synthetic and natural reference materials. In addition, nine of these species were investigated in terms of their alliinase activity. Alliinase (EC 4.4.1.4) catalyzes the conversion of odorless (+)-*S*-alk(en)yl-L-cysteine sulfoxides into volatile thiosulfinates. Cysteine sulfoxides as well as alliinase activity were found in all investigated samples, and (+)-*S*-methyl-L-cysteine sulfoxide was most abundant. (+)-*S*-Propyl-L-cysteine sulfoxide was detected in only a few, not closely related, species. Analysis of the crude protein extract of nine species gave evidence that alliinase activities of samples were similar in terms of pH and temperature optimum, K_M value, and substrate specificity. For all investigated protein extracts, the highest specific alliinase activity was found for (+)-*S*-(2-propenyl)-L-cysteine sulfoxide (alliin). The substrate specificity of these enzymes was not related to relative abundance of the cysteine sulfoxides. However, SDS−PAGE yielded some significant differences among species in terms of their total protein compositions. Species belonging to different subgenera exhibited a specific protein pattern with molecular masses between 13 and 35 kDa.

Keywords: *Allium; Alliaceae; flavor precursors; cysteine sulfoxides; aliinase; chemotaxonomy*

INTRODUCTION

Since ancient times, many *Allium* species have been used as foods, spices, and herbal remedies in widespread areas of the world. Sulfur-containing volatile flavor compounds are responsible for the characteristic smell and taste of these species. The volatile substances are formed by the action of the enzyme alliinase (EC 4.4.1.4) when plant material is disrupted and are presented in Figure 1 (Sendl, 1995; Koch and Lawson, 1996; Breu, 1996). Mainly intact bulbs contain the odorless, nonvolatile precursors such as the alkyl- and alkenyl-substituted L-cysteine sulfoxides **1**, **3**, **4**, and **6**. Only the L-(+)-isomers of these substances have been found in nature (Koch and Lawson, 1996). For most recently investigated species, (+)-*S*-methyl-L-cysteine sulfoxide (methiin, **1**) has been reported for a number of them, for example, *A. sativum*, *A. cepa*, *A. porrum*, and *A. ursinum* (Knobloch et al., 1991; Koch and Lawson, 1996). Moreover, (+)-*S*-propyl-L-cysteine sulfoxide (**3**) and (+)-*S*-(2-propenyl)-L-cysteine sulfoxide (alliin, **4**) are characteristic of *A. porrum* and *A. sativum*, respectively. (+)-*S*-(*trans*-1-Propenyl)-L-cysteine sulfoxide (isoalliin, **5**) is the precursor of the lachrymatory factor of *A. cepa* (Block, 1992; Koch and Lawson, 1996; Breu, 1996). The ethyl and butyl homologous **2** and **6** have never been reported for any *Allium* species. Some species, which have been used as foods or spices like those mentioned above, have been extensively investigated in terms of their sulfur-containing compounds and alliinase activity (Kazaryan and Goryachenkova, 1978; Nock and Mendel, 1986; Won and Mazelis, 1989; Jansen et al., 1989; Knobloch et al., 1991; Block et al., 1992; Lohmüller et al., 1994; Landshuter et al., 1994; Rabinkov et al., 1994; Koch and Lawson, 1996).

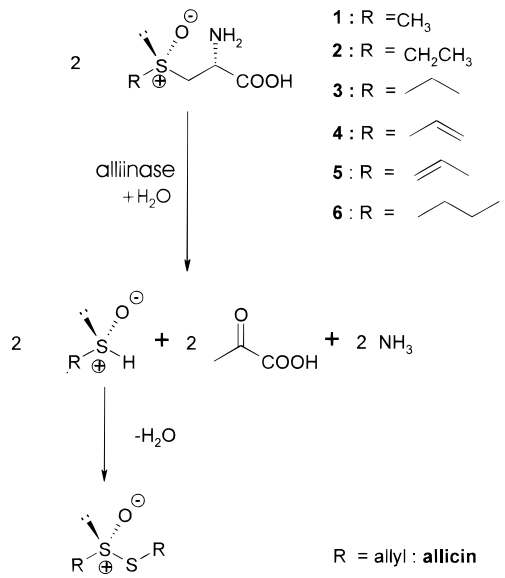

Figure 1. Alliinase-catalyzed conversion of cysteine sulfoxides **1**−**6** to the corresponding thiosulfinates. Allicin (R_3 = allyl) is enzymatically formed from two molecules of alliin (**4**, R_1 = allyl).

As shown in Figure 1, two molecules of cysteine sulfoxides are needed to form one molecule of volatile thiosulfinate via the corresponding sulfenic acid. Due to the fact that most species contain a variety of cysteine sulfoxides, the number of resulting thiosulfinates is even higher. Freeman and Whenham (1975), the first to investigate the volatile flavor components of a number of *Allium* species, reported significant differences in the compositions of thiosulfinates. The complete thiosulfin-

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10.1021/jf990521+ CCC: $19.00
Published on Web 07/15/2000

ate pattern of garlic homogenates is described by Lawson et al. (1991), but it must be considered that thiosulfinates are relatively unstable. Storage over a longer period or steam distillation resulted in di-, tri-, and polysulfides, ajoenes, and vinyldithiines. Overviews of these complex reactions were given by Koch and Lawson (1996), Block (1992), and Winkler et al. (1991). These organosulfur compounds were recently described for a CO_2 extract of *Allium tricoccum* (Calvey et al., 1998) or as constituents of the volatile oil of an interspecific hybrid of onion and leek (Schulz et al., 1998).

In contrast to these investigations, we studied the flavor precursors of 14 different wild *Allium* species and a Chinese variety of *A. sativum*. The investigated plants belong to the subgenera *Allium*, *Rhizerideum*, *Amerallium*, and *Melanocrommyum*. The sample of *A. sativum* was chosen because Chinese garlic is used not only for the production of garlic powder but also for phytotherapeutic medications. *Nectaroscordum tripedale* and *Tulbaghia acutiloba*, two additional species of the Alliaceae family, were also analyzed.

However, a problem of this type of investigation is significant variations of the cysteine sulfoxide content over the period of vegetation (Koch and Lawson, 1996; Sticher and Mütsch-Eckner, 1991). Hence, all samples of bulbs and rhizomes analyzed in the present study were harvested in the dormant phase and were immediately screened for substances **1**−**6** by HPLC**.** Because a sufficiently active alliinase is indispensable for the formation of thiosulfinates, the enzymatic activity of an extracts was studied in view of its substrate specificity.

To the best of our knowledge, this is the first study about a simultaneous determination of flavor precursors and alliinase activity. This allows a direct correlation between results obtained by HPLC and enzymatic analysis. This correlation is important for the decision of which wild *Allium* species are suitable for breeding experiments to create new hybrids. These hybrids may be valuable as crops (mild taste; low content of alliin and/or isoallinn) or spices (hot taste; high content of cysteine sulfoxides).

MATERIALS AND METHODS

Reagents. All chemicals unless otherwise specified were purchased from Merck or Fluka and were purified by standard procedures if necessary (distillation of solvents). Standards of molecular masses were obtained from Promega (Madison, WI; Ord.-no. V 5231), and polyacrylamide as well as Bradford reagent was purchased from Sigma.

Plant Material. Samples of *A. sphaerocephalon* (globe garlic, drumsticks), *A. hymenorrhizum*, *A. saxatile*, *A. obliquum*, *A. subhirsutum*, *A. jesdianum*, and *A. stipitatum* were obtained from the Institute for Plant Genetics and Crop Plant Research, Gatersleben, Germany. *A. vineale* (field garlic) and *A. triquetrum* (three-cornered garlic), *A. paradoxum* (few flowered garlic), *A. carinatum* (keeled garlic), and *Nectaroscordum tripedale* (false garlic) were gifts from the Botanical Gardens of the Universities of Cologne, Frankfurt, Dresden, and Leipzig (Germany), respectively. Species were collected in 1997 and were cultivated in the institute garden before further use (outdoor cultivation). *A. oleraceum* (wild onion), *A. victorialis* (alpine leek), and *A. ursinum* (wild garlic, ramsons) were grown outdoors in the garden of the Institute for Pharmaceutical Biology, Bonn, Germany. A sample of fresh *A. sativum* (garlic) was a gift from Prof. Fengsheng Zhao, University of Shanghai, People's Republic of China. *Tulbaghia acutiloba* (wild garlic) was obtained from Prof. A. E. van Wyk, University of Pretoria, South Africa.

Sample Preparation. Rhizomes and bulbs were all harvested at the same time in January (with the exception of a sample of *A. sativum*, which was freshly obtained from China) and processed immediately; all were treated in the same manner. For HPLC analysis, 0.2−0.8 g of an exactly weighed sample (one to five bulbs and/or rhizomes) were heated for 10 min in 20 mL of MeOH under reflux and then crushed in a mortar and returned to the MeOH for further extraction by the addition of 20 mL of H_2O. The extract was filtered, and the residue was washed with portions of 3 mL of MeOH. The combined filtrates were carefully evaporated under vacuum to dryness and stored at −20 °C before further use. Shortly before analysis, the residue was resolved in the derivatization reagent to give a final volume of 5.00 mL. Volumes of 15 μL were analyzed after 30 min of incubation in the dark. The derivatization reagent was prepared as follows: 140 mg of *o*-phthaldialdehyde (OPA) was dissolved in 5 mL of MeOH, and 0.2 mL of 2-methylpropanethiol was added. The reagent was mixed and diluted by 50 mL of 0.05 M sodium tetraborate buffer (pH 9.5). The solution was prepared 12 h before first use (Ziegler and Sticher, 1989).

For enzyme analysis, 1.5−3 g (fresh weight) of cleaned and peeled samples was crushed in an ice-cold mortar and extracted for 5 min at 4 °C with 10 mL of phosphate buffer (60 mM, pH 7.0) containing 1% NaCl, 10% sucrose, 25 mM P-5′-P, and 0.02% NaN_3. The extract was centrifuged for 30 min at 11500*g*. The supernatant was filtered and dialyzed overnight at 8 °C against the same buffer described above (Servapor dialysis tubing, 21 mm diameter, protein exclusion at 10 kDa, stirred buffer reservoir).

HPLC Analysis. Quantitative analysis was performed either on a Shimadzu LC-4A chromatograph equipped with a Chromatopac C-R3A integrator (gradient solvent system) or on a system consisting of a Knauer 64 pump, a Knauer UV detector, and a Spectra Physics SP 4400 integrator (isocratic solvent system). A Spherimarge 80-ODS2 RP column (5 μm particle size; 250 × 4 mm with integrated guard column) was operated at a flow rate of 1.0 mL/min throughout. Detection was carried out at 335 nm.

Best results were achieved by using a modified procedure first described by Ziegler and Sticher (1989). A gradient starting at 21% acetonitrile in phosphate buffer (pH 6.5, 0.05 M), 24% acetonitrile between 10 and 20 min, up to 27% at 40 min and 30% after 45 min was most suitable (method 1). For an isocratic solvent system, a mixture of acetonitrile and phosphate buffer (27:73) was alternatively used (method 2). Peaks were identified by cochromatography with standards. Synthesized material was applied with the exception of **5**, which was obtained from an authentic sample of *A. cepa* L. (Breu, 1996). Calibration with **1**, **3**, and **4** was carried out over a range of 0.01−0.4 mg/mL. Standards were derivatized in the same way as described above. Because **4** and **5** are isomers showing the same calibration plot, concentrations of both substances were determined by the calibration plot of **4**. HPLC runs of samples were repeated three times.

Reference material was synthesized following the procedure formerly described (Theodoropoulus, 1991; Stoll and Seebeck, 1951; Iberl et al., 1990; Freeman and Huang, 1994). Racemic sulfoxides **1**−**4** and **6** were prepared from L-cysteine by sulfur alkylation followed by oxidation of the sulfur atom. Diastereomers of sulfoxides were separated by fractional recrystallization using H_2O/acetone or H_2O/alcohol mixtures. All fractions were subjected to TLC and HPLC (Ziegler and Sticher, 1989; Keusgen, 1997). Synthesized reference materials showed a purity of at least 95%.

Analytical Data. For EI-MS, a MS 50 (Kratos) was operated at 70 eV. FAB mass spectra were obtained on a Concept 1H (Kratos), Xe gun, by using 3-nitrobenzyl alcohol or glycerol as matrix. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) were recorded by a Varian XL-300 spectrometer. IR spectra were obtained on a Perkin-Elmer 720 spectrometer. A disk was prepared from 0.7 mg of sample and 300 mg of KBr.

*Analytical Data of (+)-S-Methyl-L-cysteine Sulfoxide (**1**):* IR (KBr) ν_{max} 3420 (H_2O), 2960 ($-CH_3$, $-CH_2-$), 1620 (NH_3^+,

COO^-), 1460 (NH_3^+), 1350 (−O−H), 1050, 980 (S−O), 820 cm^{-1}; ^1H NMR (D_2O, CD_3OD 8:2) δ 4.20 (1H, dd, J = 8, 6 Hz, H-2), 3.23 (1H, dd, J = 13, 8 Hz, H-3), 3.49 (1H, dd, J = 13, 6 Hz, H-3), 2.84 (3H, *s*, H-4); ^{13}C NMR (D_2O, CD_3OD 8:2) δ 173.9 (C-1), 53.8 (C-2), 55.9 (C-3), 41.0 (C-4); FABMS, *m*/*z* 152.0 $[MH]^+$ for $C_4H_{10}O_3NS$; t_R (HPLC, method 1) 28.1 min.

*Analytical Data of (+)-S-Ethyl-L-cysteine Sulfoxide (**2**):* IR (KBr) ν_{max} 3420 (H_2O), 2950 ($-CH_3$, $-CH_2-$), 1620 (NH_3^+, COO^-), 1480 (NH_3^+), 1380 (−O−H), 995 (S−O) cm^{-1}; ^1H NMR (D_2O, CD_3OD 8:2) δ 4.20 (1H, dd, J = 8, 6 Hz, H-2), 3.19 (1H, dd, J = 14, 8 Hz, H-3), 3.45 (1H, dd, J = 14, 6 Hz, H-3), 2.98 (1H, m, J = 13, 8 Hz, H-4), 3.07 (1H, m, J = 13, 8 Hz, H-4), 1.35 (3H, t, J = 8 Hz, H-5); ^{13}C NMR (D_2O, CD_3OD 8:2) δ 174.0 (C-1), 53.9 (C-2), 53.1 (C-3), 48.7 (C-4), 8.6 (C-5); FABMS, *m*/*z* 166.0 $[MH]^+$ for $C_5H_{12}O_3NS$.

*Analytical Data of (+)-S-Propyl-L-cysteine Sulfoxide (**3**):* IR (KBr) ν_{max} 3420 (H_2O), 2950 ($-CH_3$, $-CH_2-$), 1630 (NH_3^+, COO^-), 1520 (NH_3^+), 1320 (−O−H), 1050, 995 (S−O), 820 cm^{-1}; ^1H NMR (D_2O, CD_3OD 8:2) δ 4.23 (1H, dd, J = 8, 6 Hz, H-2), 3.22 (1H, dd, J = 14, 8 Hz, H-3), 3.47 (1H, dd, J = 14, 6 Hz, H-3), 2.97 (1H, m, J = 13, 7, 2 Hz, H-4), 3.00 (1H, m, J = 13, 8, 4 Hz, H-4), 1.75−1.88 (2H, m, H-5), 1.08 (3H, t, J = 8 Hz, H-6); ^{13}C NMR (D_2O, CD_3OD 8:2) δ 174.0 (C-1), 53.8 (C-2), 56.8 (C-3), 53.7 (C-4), 18.5 (C-5), 15.1 (C-6); FABMS, *m*/*z* 180.1 $[MH]^+$ for $C_6H_{14}O_3NS$; t_R (HPLC, method 1) 49.7 min.

*Analytical Data of (+)-S-(2-Propenyl)-L-cysteine Sulfoxide [**4**, L-(+)-Alliin]:* analytical Data are given by Sendl (1995) and Koch and Keusgen (1998); t_R (HPLC, method 1) 27.0 min.

Analytical Data (−)-S-(2-Propenyl)-L-cysteine Sulfoxide [L-(−)-Alliin]: IR (KBr) ν_{max} 3420 (H_2O), 3060 (NH_3^+), 2960 ($-CH_2-$), 1620 (NH_3^+, COO^-), 1495 (NH_3^+), 1380 (−O−H), 985 (S−O), 900 (C=C−H), 820 cm^{-1}; ^1H NMR (D_2O, CD_3OD 8:2) δ 4.23 (1H, dd, J = 8, 4 Hz, H-2), 3.36 (1H, dd, J = 13, 8 Hz, H-3), 3.43 (1H, dd, J = 13, 4 Hz, H-3), 3.70 (1H, m, J = 13, 8, 1 Hz, H-4), 3.85 (1H, m, J = 13, 7, 1 Hz, H-4), 5.94 (1H, m, J = 17, 10, 8, 7 Hz, H-5), 5.52 (1H, m, J = 17, 1 Hz, H-6), 5.56 (1H, m, J = 10, 1 Hz); ^{13}C NMR (D_2O, CD_3OD 8:2) δ 173.7 (C-1), 53.0 (C-2), 57.4 (C-3), 52.2 (C-4), 127.1 (C-5), 127.9 (C-6); FABMS, *m*/*z* 178.0 $[MH]^+$ for $C_6H_{12}O_3NS$; t_R (HPLC, method 1) 45.9 min.

*Analytical Data of (+)-S-(trans-1-Propenyl)-L-cysteine Sulfoxide (**5**, Isoalliin):* isolation, HPLC analysis, and analytical data are given in Breu (1996) and Carson et al. (1966); t_R (HPLC, method 1) 47.3 min.

*Analytical Data of (+)-S-Butyl-L-cysteine Sulfoxide (**6**):* IR (KBr) ν_{max} 3420 (H_2O), 2940 ($-CH_3$, $-CH_2-$), 1580 (NH_3^+, COO^-), 1380 (−O−H), 1050, 995 (S−O), 820 cm^{-1}; ^1H NMR (D_2O, CD_3OD 8:2) δ 4.22 (1H, dd, J = 8, 6 Hz, H-2), 3.20 (1H, dd, J = 14, 8 Hz, H-3), 3.46 (1H, dd, J = 14, 6 Hz, H-3), 2.94−3.10 (2H, m, H-4), 1.70−1.81 (2H, m, H-5), 1.41−1.59 (2H, m, H-6), 0.96 (3H, t, J = 7 Hz, H-7); ^{13}C NMR (D_2O, CD_3OD 8:2) δ 173.8 (C-1), 53.9 (C-2), 54.7 (C-3), 53.7 (C-4), 26.7 (C-5), 24.0 (C-6), 15.6 (C-7); FABMS, *m*/*z* 194.1 $[MH]^+$ for $C_7H_{16}O_3NS$.

Enzyme Activity, Protein Characterization. Protein concentrations of alliinase preparations were determined following the methods described by Lowry et al. (1951) using BSA as standard. Enzyme activity was calculated from the amount of enzymatically formed pyruvate as published by Schwimmer and Mazelis (1963, see Figure 1). The standard reaction mixture contained 18 units of lactate dehydrogenase, 0.4 μmol of NADH, and 4 μmol of L-(+)-alliin (**4**). Volume was adjusted with phosphate buffer (60 mM, pH 7.0) to give 1.00 mL. The decrease of NADH was traced at 340 nm. Standard experiments were performed at 25.0 °C.

Substrate specificity of alliinase was determined analogously. Activity toward racemic **4** was set to 100%. Compounds **1**−**3** and **6** were used as racemates, and enzyme activity was related to racemic **4**. For determination of enzyme activity at different pH values, phosphate buffer (60 mM, pH 7.0) was replaced by a citric acid/phosphate buffer (0.2 M) in the range pH 3−7, phosphate buffer (0.2 M) in the range pH 6−8, and borate buffer (0.2 M) in the range pH 7.5−11. Temperature optimum of the alliinase was investigated in a range from 20 to 56 °C in steps of 4 °C. K_M and V_{max} values were deduced from a Lineweaver−Burk plot. Sulfoxide **4** [L-(+)-diastereomer] in concentrations from 0.04 to 4 mM was used as substrate. Protein samples (20−30 μg of protein) were also subjected to dissociating sodium dodecyl sulfate gel electrophoresis (SDS−PAGE) as published by Laemmli (1970). A polyacrylamide concentration of 10% was used throughout all experiments. Proteins were visualized by staining with Coomassie blue, and masses were determined by calibration proteins.

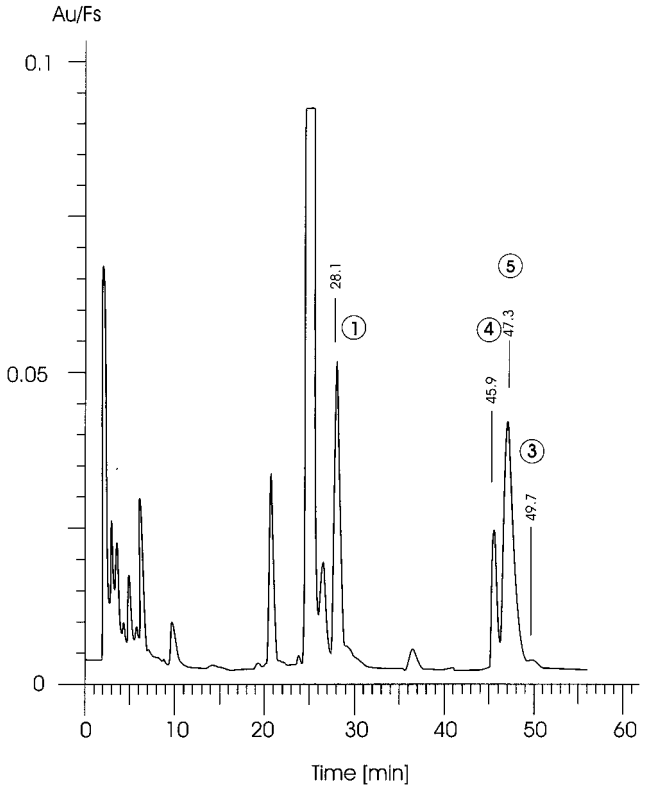

Figure 2. HPLC chromatogram of the methanolic extract obtained from *A. vineale*. Cysteine sulfoxides were derivatized with the OPA reagent and are labeled according to Figure 1.

RESULTS

Cysteine Sulfoxides. Determination of cysteine sulfoxides was carried out by means of HPLC analysis. With the exception of **5**, reference material was synthesized at the beginning of the study. Structures were confirmed by means of NMR, MS, and IR analysis. In agreement with previous investigations (Koch and Lawson, 1996), only the L-(+)-diastereomers of **1** and **3**−**5** were found as naturally occurring cysteine sulfoxides (Figure 2). Results and the taxonomic classification of investigated species are listed in Table 1. The relative composition of cysteine sulfoxides is shown in Figure 3.

Substance **1** was detected in all samples. As expected, highest amounts were found in the genus *Allium*. Most species with the exception of *A. sativum*, *A. sphaerocephalon*, *A. vineale*, and *A. obliquum* did contain **1** as the major component. On the other hand, low amounts of **3** were detected in only a few, not closely related, species (*A. vineale*, *A. victorialis*, *A. ursinum*, *A. triquetrum*, and *T. acutiloba*).

Five of the investigated *Allium* species belong to the subgenus *Allium* (I), which is divided into sections Ia and Ib (*Allium* and *Codonoprasum*, respectively). The occurrence of **1** and **5** seems to be typical for the whole subgenus I. In contrast, **4** was detected only in section Ia and can be probably used for differentiation between Ia and Ib. The total amount of sulfoxides was relatively

Table 1. Taxonomic Classification and Cysteine Sulfoxides 1 and 3−5 of Different Species Belonging to the Alliaceae Family[a]

section/ subgenus	species	total (%)	**1** (%) (methyl-)	**4** (%) (2-propenyl-)	**5** (%) (1-propenyl-)	**3** (%) (propyl-)
	Genus *Allium* L., Subgenus *Allium* L.					
Ia	*A. sativum* L. (bulb)	0.229	0.033	0.173	0.023	nd
Ia	*A. sphaerocephalon* L. (bulb)	0.050	0.018	0.011	0.021	nd
Ia	*A. vineale* L. (bulb)	0.182	0.047	0.032	0.102	0.001
Ib	*A. carinatum* L. (bulb)	0.625	0.487	nd	0.138	nd
Ib	*A.oleraceum* L. (bulb)	0.154	0.144	nd	0.010	nd
	Genus *Allium* L., Subgenus *Rhizerideum* (G. Don ex Koch) Wbd.					
IIa	*A. victorialis* L. (bulb)	0.104	0.078	0.026	nd	nd
IIa	*A. victorialis* L. (rhizome)	0.117	0.063	0.052	nd	0.002
IIb	*A. hymenorrhizum* Lebedour (bulb)	0.057	0.045	0.001	0.011	nd
IIb	*A. saxatile* M. Bieb. (bulb)	0.661	0.623	0.012	0.026	nd
IIc	*A. obliquum* L. (bulb)	0.064	0.028	0.036	nd	nd
	Genus *Allium* L., Subgenus *Amerallium* Traub					
IIIa	*A. ursinum* L. (bulb)	0.309	0.163	0.060	0.084	0.002
IIIb	*A. paradoxum* (M. Bieb.) G. Don (bulb)	0.040	0.032	n.d.	0.008	nd
IIIb	*A. triquetrum* L. (bulb)	0.128	0.081	0.018	0.020	0.009
IIIc	*A. subhirsutum* L. (bulb)	0.033	0.026	0.007	nd	nd
	Genus *Allium* L., Subgenus *Melanocrommyum* (Webb et Berth.) Rouy					
IVa	*A. jesdianum* ssp. *angustitepalum* (Wendelbo) Khassanov et Fritsch (bulb)	0.062	0.060	nd	0.002	nd
IVa	*A. stipitatum* Regel (bulb)	0.044	0.043	nd	0.001	nd
	Genus *Nectaroscordum* Lindl.					
	N. tripedale (Trautv.) Grossh. (bulb)	0.010	0.010	nd	nd	nd
	Genus *Tulbaghia* L.					
	T. acutiloba Harv. (rhizome)	0.006	0.004	nd	0.001	0.001

[a] Concentrations related to fresh weight. The genus *Allium* is divided into subgenera (I−IV) and sections, which are as follows: Ia, *Allium* L.; Ib, *Codonoprasum* Reichenb. in Mössl.; IIa, *Anguinum* G. Don. ex Koch; IIb, *Oreiprason* F. Herm.; IIc, *Petroprason* F. Herm.; IIIa, *Arctoprasum* Kirschl.; IIIb, *Briseis* (Salisb.) Stearn; IIIc, *Molium* G. Don ex Koch; IVa, *Megaloprason* Wendelbo s. str. (Fritsch, 1993).

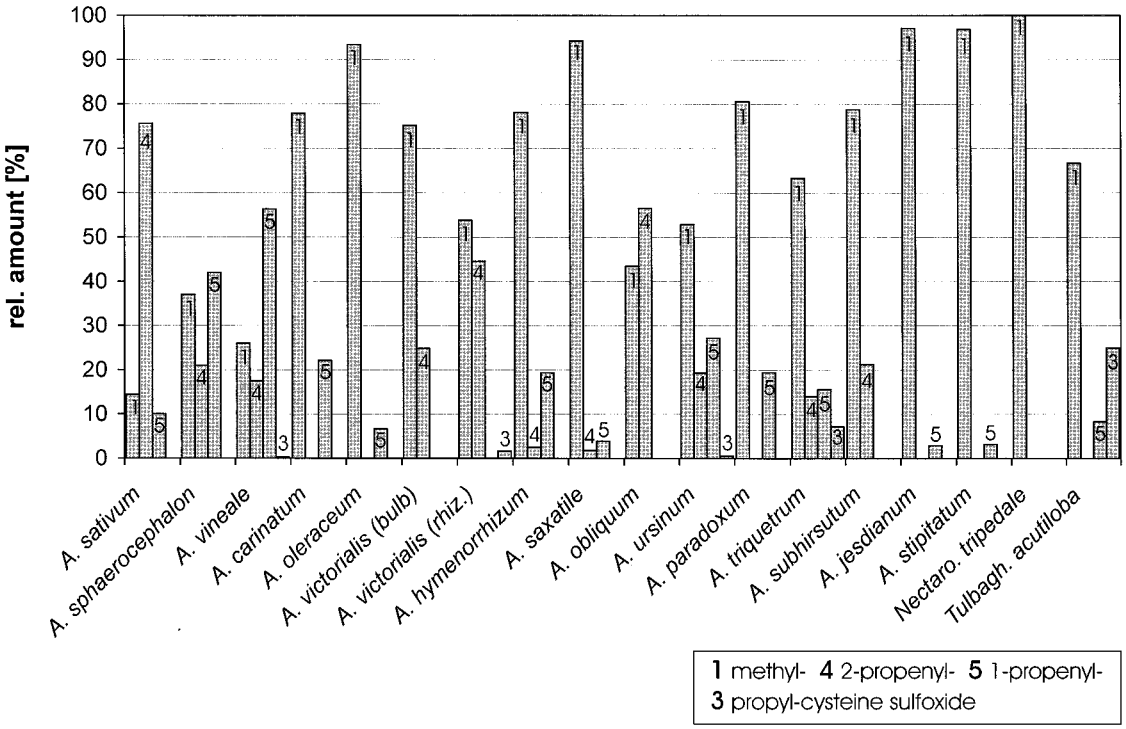

Figure 3. Relative amounts of cysteine sulfoxides **1** and **3**−**5** of different Alliaceae (total cysteine sulfoxides = 100%).

high (with the exception of *A. sphaerocephalon*). The closely related *A. sphaerocephalon* and *A. vineale* also exhibited a very similar sulfoxide pattern (Figure 3).

Sulfoxides **1** and **4** were found throughout the subgenus *Rhizerideum* (II); **5** occurred in *A. hymenorrhizum* and *A. saxatile*, both belonging to section IIb (*Oreiprason*). Concentration of cysteine sulfoxides of *A. victorialis* L., section IIa (*Anguinum*), was different in the bulb and in the rhizome parts of the same plant. The rhizome additionally showed low amounts of **3**. The composition of cysteine sulfoxides is similar to that of *A. obliquum* (section IIc, *Petroprason*).

Sections IIIa−c (*Arctoprasum*, *Briseis*, and *Molium*, respectively) are part of the subgenus *Amerallium* (III).

Table 2. Characteristics of Crude Alliinase Preparations Obtained from Some Selected *Allium* Species

species	pH activity range	pH at half-V_{max}	pH optimum	temp optimum (°C)	K_M (mM)	V_{max} (μmol min^{-1} mg^{-1})	protein subunits (kDa), decreasing intensity
A. sativum	4.5−10.0	5.5, 8.5	7.0	40	1.25	178	53, 28, 39, 38
A. sphaerocephalon	4.5−8.5	5.5, 8.0	7.0	36	1.59	6	29,53, 54, 56, 57, 39, 41
A. victorialis	4.5−9.5	5.5, 8.5	7.5	44	0.42	3	25, 29,52, 54, 56, 41
A. hymenorrhizum	4.5−9.0	6.0, 8.5	7.5	36	1.48	3	26, 17, 29, 30, 31, 54, 56
A. saxatile	4.5−9.5	5.5, 8.5	7.5	38	0.87	18	25, 32, 33, 29, 53, 15
A. obliquum	4.5−9.0	5.5, 8.5	7.5	38	0.77	13	26, 32, 17, 53, 28, 27
A. subhirsutum	4.5−9.5	6.5, 8.5	7.5	40	0.85	7	22, 24, 28, 54
A. jesdianum	5.0−9.0	6.5, 8.5	7.5	48	1.79	2	30, 32, 35, 26, 55
A. stipitatum	4.5−9.5	5.5, 8.5	7.5	38	0.68	7	32, 26, 25, 52, 54, 56

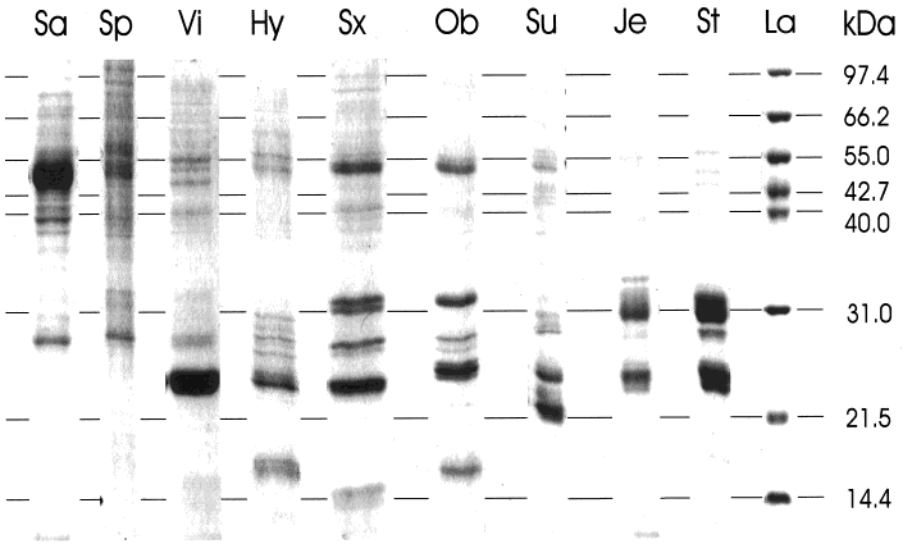

Figure 4. SDS−PAGE of protein fractions obtained from various *Allium* samples. Subunits of alliinase are expected to have a molecular mass of 48−56 kDa. Sa, *A. sativum*; Sp, *A. sphaerocephalon*; Vi, *A. victorialis*; Hy, *A. hymenorrhizum*; Sx, *A. saxatile*; Ob, *A. obliquum*; Su, *A. subhirsutum*; Je, *A. jesdianum*; St, *A. stipitatum*; La, protein standards.

Investigated species of III exhibited an irregular cysteine sulfoxide pattern. *A. ursinum* and *A. triquetrum* showed a similar qualitative composition (**1**, **3**, **4**, and **5**). The two other species of this subgenus are characterized by a relatively high amount of **1** and traces of **4** and **5** (*A. subhirsutum* and *A. paradoxum*, respectively).

A. jesdianum and *A. stipitatum* are members of the subgenus *Melanocrommyum* (IV), section IVa (*Megaloprason*). Similar concentrations for **1** and **5** were found for both species. *N. tripedale* and *T. acutiloba* showed very low amounts of cysteine sulfoxides. Again, **1** was obtained in both species. Surprisingly, traces of **3** and **4** were found in the rhizome of *T. acutiloba*.

Alliinase Activity. As shown in Figure 1, action of alliinase is necessary to convert odorless cysteine sulfoxides into characteristic smelling thiosulfinates. Therefore, an aqueous extract of some representative species was prepared, and alliinase activity was investigated. Results of protein analysis are listed in Table 2. All enzyme extracts exhibited alliinase activity in a pH range between 5 and 8. The temperature optimum for most alliinases was found between 36 and 40 °C. Alliinase from *A. victorialis* (extract of bulb and rhizome) and *A. jesdianum* was still active at higher temperatures (44 and 48 °C, respectively). K_M values were in the range between 0.42 mM (*A. victorialis*) and 1.79 mM (*A. jesdianum*).

In addition, V_{max} of enzyme extracts was estimated. Highest specific alliinase activity was found for *A. sativum* (178 μmol min^{-1} mg^{-1}). Also, samples of *A. saxatile* and *A. obliquum* (both subgenus II) showed a relatively high alliinase activity (18 and 13 μmol min^{-1} mg^{-1}, respectively). All other samples exhibited a V_{max} < 9 μmol min^{-1} mg^{-1}.

In previous studies with purified alliinase, mainly obtained from culture varieties, molecular masses of subunits were reported to be in a range between 48 and 54 kDa (Kazaryan and Goryachenkova, 1978; Nock und Mendel, 1986; Won and Mazelis, 1989; Jansen et al., 1989; Lohmüller et al., 1994; Landshuter et al., 1994; Rabinkov et al., 1994). At least two of these subunits are necessary for the active holoenzyme. Results from SDS−PAGE are shown in Figure 4. As expected, the electropherogram of the extract of *A. sativum* shows an intense band at 53 kDa, which is in accordance with a relatively high enzyme activity. Also, samples from *A. saxatile* and *A. obliquum* contain considerable amounts of a 53 kDa protein, which was suggested to be an alliinase subunit. All other samples showed a low intensity of bands in the range between 48 and 54 kDa, being also in accordance with the low alliinase activity of these extracts. In this range, *A. stipitatum* and *A. jesdianum* gave protein bands of very low intensity.

Relative enzyme activities toward different cysteine sulfoxides are depicted in Figure 5. The activity toward racemic **4** [L-(±)-alliin] was set to 100% because synthetic **1**−**3** and **6** could not be obtained as pure L-(+)-isomers and were therefore used as their racemates. In addition, enzyme activities for pure **4** and for the unnatural L-(−)-isomer were determined. All samples showed highest activity toward **4**. The L-(−)-isomer is enzymatically converted at much lower rates. Alliinase activity of the protein extract obtained from *A. sphaero-*

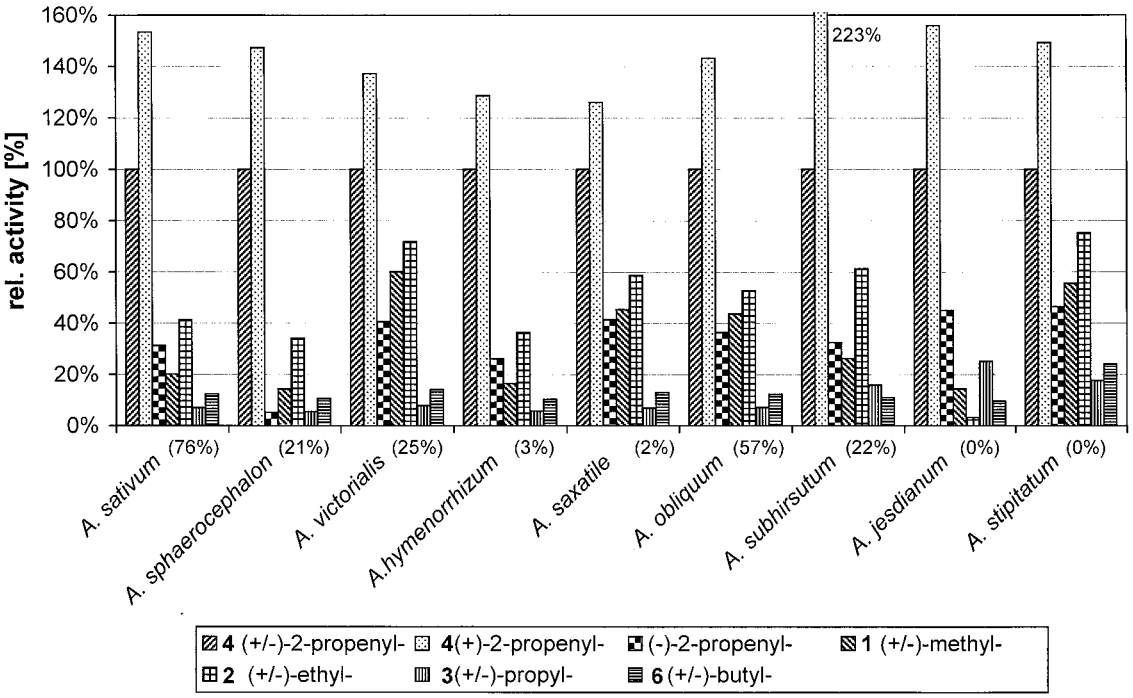

Figure 5. Relative alliinase activity of protein extracts of some selected *Allium* species. Activity toward (±)-alliin **4** was set to 100%. Relative alliin concentrations according to Figure 3 are given in parentheses below each set of columns.

cephalon was most stereoselective, whereas alliinase activity of *A. victorialis* and *A. stipitatum* was most unspecific. The sample obtained from *A. subhirsutum* gave the highest relative activity toward pure **4**.

Further Proteins. In addition, analysis of proteins with a low molecular mass may be useful for the taxonomic classification of species. With the exception of the species belonging to the subgenus *Allium*, all samples exhibited proteins with molecular masses between 13 and 27 kDa. Species of the sections IIb and IIc additionally showed considerable amounts of a 15−17 kDa protein (*A. hymenorrhizum*, *A. saxatile*, and *A. obliquum*). *A. subhirsutum* (IIIc) showed an intense band at 22 kDa. The protein patterns of *A. jesdianum* and *A. stipitatum* (IVa) were very similar and are characterized by several intense bands in the range between 25 and 32 kDa.

DISCUSSION

It has been demonstrated that the composition of cysteine sulfoxides can differ significantly among species belonging to the family of Alliaceae. Compound **1** is least abundant and probably characteristic of the whole family; compound **3** is less abundant and seems to occur in considerable amounts only in a limited number of species. Cysteine sulfoxide **4** occurred in the subgenera *Allium*, *Rhizerideum*, and *Amerallium* in considerable amounts.

The composition of sulfur-containing compounds of *A. sativum* and *A. ursinum* was also analyzed in recent studies (Freemann et al., 1975; Ziegler and Sticher, 1989; Lawson et al., 1990; Block et al., 1992; Sendl, 1995; Koch and Lawson, 1996). In agreement with these studies, it could be demonstrated that *A. sativum* mainly contains **4**. Substances **1** and **5** occurred in much lower amounts. In recent studies, compound **4** was found in a concentration range between 0.1 and 1.2% (Ziegler and Sticher, 1989), so the investigated Chinese sample probably belongs to a mild garlic variety. Nevertheless, *A. sativum* showed the highest amount of **4** of all investigated species.

Cysteine sulfoxides **1**, **3**, and **4** were also reported for *A. ursinum* (Freeman et al., 1975; Sendl, 1995). Investigations carried out by HPLC demonstrated that **1** and **4** were present in nearly equal amounts (Sendl, 1995). We found that **1** is the main cysteine sulfoxide of *A. ursinum*. In contrast to these studies, low concentrations of **5** were found (<0.1%). This difference may be explained by the improved HPLC system, by which the isomers **4** and **5** were separated. Also, Block et al. (1992) described small amounts of thiosulfinates from *A. ursinum* containing a 1-propenyl moiety, which may be deduced from **5** after the action of alliinase.

Alliinase degradation products of *A. vineale* were even analyzed by Freeman et al. (1975). In agreement with this study, compounds **1**, **3**, and **4** were suggested to be characteristic for this species. However, only traces of **5** have been detected in the former study, which is in contrast with the present study. Freeman also analyzed *Nectaroscordum siculum* (Ucria) Lindley, a species that is closely related with the now investigated *N. tripedale*. In agreement with this study, **1** was suggested as the main cysteine sulfoxide.

Because of their relatively high content of cysteine sulfoxides, wild *Allium* species belonging to the subgenus *Allium* and the subgenus *Rhizerideum* (e.g., *A. carinatum* and *A. saxatile*) may be suitable for breeding of new crop plants. This is in good agreement with the fact that a number of crop plants such as *A. cepa* and *A. porrum* also belong to these subgenera. In addition, *A. ursinum* and *A. triquetrum* may be of interest because of their sulfoxide composition, but the total amount of cysteine sulfoxides should be increased by plant breeding. On the other hand, species belonging to the subgenus *Melanocrommyum* are less suitable as a spice but can be probably used for breeding of mild smelling vegetables.

Species with a high content of cysteine sulfoxides, especially with a high content of **4**, are also suitable for breeding of new medicinal plants. Is has been shown that alliin-derived compounds are responsible for the health benefits of garlic (Koch and Lawson 1996). Further sulfur compounds are also pharmacologically active (Block, 1992; Koch and Lawson, 1996).

The formation of a variety of different thiosulfinates and the corresponding transformation products always depends on the action of alliinase. Hence, sufficient enzyme activity is necessary for a useful crop or medicinal plant. High alliinase activities were found in the subgenera *Allium* and *Rhizerideum*; lowest activity was found in the subgenus *Melanocrommyum*. These results correlated with the absolute amount of cysteine sulfoxides of the corresponding species.

It must be considered that the content of cysteine sulfoxides of individual species may vary over the vegetation period (Koch and Lawson, 1996; Sticher and Mütsch-Eckner, 1991). Due to the limited number of samples for this study, this question cannot be answered for the now investigated species. Nevertheless, this study allows a good comparison of individual species because they were all harvested at the same time and processed in an identical way. The relative composition of cysteine sulfoxides seems to be more stable over the year than the absolute amount (Koch and Lawson, 1996).Therefore, this study can be used for planning hybridization experiments to breed new species that are suitable as vegetables and spices.

ACKNOWLEDGMENT

We thank students, who participated at the phytochemical course of the Institute for Pharmaceutical Biology during the winter of 1997/1998, for technical assistance. We are grateful to Drs. L. D. Lawson, E. Leistner, K.-W. Glombitza, and C. Drewke for helpful discussions and financial support. We also thank Dr. R. Fritsch, Prof. Dr. W. Hempel, Prof. Fengsheng Zhao, Prof. A. E. van Wyk, Dr. H. Winter, Mr. H. Zimmer, and Mr. Wessel for providing plant material. We also thank the Central Analytical Department of the Institute of Chemistry and the NMR Department of the Pharmaceutical Institute, University of Bonn, for mass and NMR spectra.

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Received for review May 24, 1999. Accepted May 9, 2000. This research was supported by the German Fonds der Chemischen Industrie and the DPhG-Stiftung as part of the Stifterverband für die Deutsche Wissenschaft.

JF990521+