Proteolytic Activity in Sunflower Seeds (*Helianthus annuus* L.)

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The crude extract of defatted meal of sunflower seeds contains at least five different exopeptidases with molecular weights of 50 000–90 000: two carboxypeptidases with an optimum pH around 5; a proline iminopeptidase and two aminopeptidases, all with an optimum pH around 8. The crude extract also contains endopeptidase activity in the alkaline (benzoyl-DL-arginine-*p*-nitroanilide as the substrate) and acid pH range (casein and hemoglobin). The carboxypeptidase activity and the alkaline endopeptidase activity of the crude extracts of whole seedlings increase during germination, whereas the imino- and aminopeptidase activities remain constant or decrease. The amount of water-soluble proteins in the defatted meal of germinated seeds is maximal around the second and third day of germination. Sunflower seed meal and its water extracts also show a remarkable milk-clotting activity.

In the seeds of Spermatophytes (seed plants) proteolytic enzymes play an important role in the mobilization of storage proteins during the first days of germination (Ashton, 1976; Ryan and Walker-Simmons, 1981). Studies on different seeds, for example, mung bean seeds [*Vigna radiata* (L.) Wilczek] (Baumgartner and Chrispeels, 1978), peanut seeds (*Arachis hypogaea* L.) (Bagley et al., 1963), barley grains (*Hordeum vulgare* L.), and Scots pine seeds (*Pinus sylvestris* L.) (Mikola, 1978), indicate that the degradation process during the early stage of reserve protein catabolism begins with the hydrolysis of the "insoluble" proteins to soluble polypeptides (catalyzed by endopeptidases in the protein bodies), followed by a hydrolysis to amino acids, catalyzed by exopeptidases (Mikola, 1978; Salmia, 1981). In cotyledons of mung beans [*V. radiata* (L.) Wilczek], hydrolysis cannot start before an endopeptidase, the so-called vicilin peptidohydrolase, is synthesized (Baumgartner and Chrispeels, 1977) and transported from the cytoplasm to the protein organelles, where the storage proteins are located within the cell (Baumgartner and Chrispeels, 1978).

How the mechanism of mobilization of the reserve proteins is controlled and whether inhibitors of endogenous proteolytic enzymes take part in it are still open questions.

Among seed plants, sunflowers are of great importance worldwide, mostly as source of dietary oil. Defatted meal of sunflower seeds is also gaining attention in the field of animal and human nutrition as a source of protein (Sosulski, 1979; Brückner et al., 1982; Belart et al., 1982). The main storage protein of sunflower seeds, "helianthinin", was isolated and characterized by Schwenke et al. (1978). The protein bodies of the cotyledons of germinated seeds also contain a proteinase activity (substrate: casein, pH 5.2) (Schnarrenberger et al., 1972). An aminopeptidase activity (substrate: Lys-*p*-nitroanilide, pH 7.6) was detected in the cytoplasm (Schnarrenberger et al., 1972), and furthermore, sunflower seeds—similar to several other seeds investigated—exhibit proteolytic activity with the typical trypsin substrate Bz-DL-Arg-*p*-nitroanilide (BAPA) (Graf and Hoagland, 1969).

The change of proteolytic activity in ungerminated seeds during the processing of oil extraction was followed by using hemoglobin as the substrate at pH 5.2 (Goldovskii and Mirzoev, 1979) or pH 3.8 (Lorenz, 1980). However, a detailed investigation and characterization of the proteases of sunflower meal are still missing. Such a study is also relevant in view of the possible technological importance of these proteases in the food industry. In this paper we present data pertaining to the proteolytic activity in extracts of defatted sunflower meal and we show that at least six different proteolytic activities are present in defatted sunflower meal. We will also report that, analogous to other extracts of seeds and plant material from the family Compositae, e.g., *Cynara cardunculus* L. (Vieira de Sá and Barbosa, 1972; Barbosa et al., 1981) and *Carthamus tinctorius* L. (Tavasolian and Shabbak, 1979), defatted meal and its water extracts show a strong milk-clotting activity.

MATERIALS AND METHODS

Seed Material. In all experiments, seeds of Italian sunflower cultivars were used (*Helianthus annuus* L. cv. Argentario), and they were stored at 4 °C.

Germination. The seeds were surface sterilized with a 7% solution of NaOCl for 2 min, then washed with ethanol for 30 s, and transferred under a sterile air stream to a 2% nutrient-free agar gel in Petri dishes (five seeds in an 8.5-cm dish containing 17.5 mL of gel prepared with distilled water). The germination was in a temperature-controlled chamber at 27 °C in the dark for 1, 2, 3, 4, 5, 6, or 7 days (humidity: 100%).

Preparation of the Defatted Meal. *Ungerminated Seeds.* Hulled sunflower seeds were ground in a mixer (Osterizer Pulsematic 16) for 45 s, and *n*-hexane (to 1 g of meal/10 mL of *n*-hexane) was added to the resulting meal. To extract the oil, this suspension was shaken at room temperature for 24 h and then filtered. After the filtered residue was washed with *n*-hexane, the meal was vacuum-dried and stored at 4 °C.

Germinated Seeds. We were not able to prepare defatted sunflower meal from germinated seeds using the procedure described above, due to the water content and the presence of polyphenolic compounds (Sodini and Canella, 1977), which are enzymatically oxidized to *o*-chinons (Sabir et al., 1974; Hurell et al., 1982). This leads to the formation of dark brown products. The following alternative procedure was used: Cold acetone (−20 °C) was added to hulled germinated seeds—20 mL to 60 seeds—and immediately ground with a mixer (Ultra-Turrax from IKA, during 1 min) and then filtered with a glass suction filter apparatus. After being washed with cold acetone, the meal was vacuum-dried and then stored at 4 °C. The proteolytic activities from these two alternative procedures are similar, indicating that the hexane extraction is not

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too deleterious for the enzymatic stability.

Preparation of the Crude Extract. Ten milliliters of phosphate buffer (50 mM, pH 7.5, 0.02% NaN_3, 1% sodium disulfite) was added to 1 g of defatted meal from ungerminated seeds and the light brown mixture was stirred at 4 °C during 90 min (lower proteolytic activity was observed in the absence of disulfite). The solution was centrifuged at 10000 rpm and 4 °C during 30 min in a Beckman Model J-21 centrifuge in order to separate the insoluble material. The clear yellow-orange supernatant, prepared shortly before use, was used as the crude extract and stored at −20 °C or +4 °C. In the case of germinated seeds 30 or 60 mL of phosphate buffer was added to 1 g of the meal.

Buffer Solutions. Michaelis' barbital sodium acetate (pH 2.6–9.4; 1/7 molar sodium acetate in 1/7 molar barbital sodium), Walpole's acetate (pH 3.6–5.4; 0.1 M), Gomoris's Tris (pH 7.2–9.0; 0.1 M), and a sodium phosphate buffer (pH 5.7–8.0; 0.05 M) were prepared and used as buffer solutions. The buffer used for extraction and chromatographic separations contained 0.02% sodium azide to prevent the growth of microorganisms.

Determination of the Protein Concentration. The protein content of the solutions was determined with the Bradford (Bio-Rad) protein assay (Bio-Rad Laboratories, 1979; Fazekas et al., 1963; Bradford, 1976); bovine serum albumin was used (30–100 μg/mL) as the standard.

Protease Assays. *p-Nitroanilides.* The hydrolysis of the *p*-nitroanilides was followed at 405 nm in 3-mL cuvettes of 1-cm path length at 30 °C (Erlanger et al., 1961, 1966; Tuppy et al., 1962; Nagel et al., 1965). The value used for ϵ_{405nm} was 9900 M^{-1} cm^{-1} (Nagel et al., 1965). For poorly soluble substrates, a concentrated stock solution in Me_2SO was occasionally utilized. Fresh substrate solutions were prepared before use; the reference cell in the spectrophotometer contained the substrate solution without enzyme.

Pro-β-naphthylamide. The formation of β-naphthylamine was followed spectrophotometrically at 340 nm (Lee et al., 1971) in 3-mL cuvettes of 1-cm path length at 30 °C. The value used for ϵ_{340nm} was 1780 M^{-1} cm^{-1}. The freshly prepared stock solution was made with Me_2SO.

Z-Phe-Phe and Z-Phe-Ala. The carboxypeptidase assay was carried out at 30 °C as described by Mikola and Kolehmainen (1972) with TNBS determination of free amino groups after hydrolysis of the N-protected dipeptides. The extinction coefficient (ε) at 340 nm used for TNP-Phe was 12300 M^{-1} cm^{-1} and for TNP-Ala was 12200 M^{-1} cm^{-1} (Satake et al., 1960). Fresh substrate solutions were prepared before use. The substrate solutions were monitored for a "blank reaction" by incubating the substrate solution without enzyme. The enzyme was added after the incubation time, immediately before the alkaline TNBS solution was added.

Leu-Tyr and Ala-Gly. The hydrolysis of these two dipeptides at 30 °C was followed with TNBS as the reagent for the determination of free amino groups, using the procedure described in the literature (Mikola and Kolehmainen, 1972; Binkley et al., 1968). For estimation of the extent of hydrolysis the extinction coefficient at 340 nm for the TNP amino acids determined by Satake et al. (1960) were used: TNP-Leu, 14200 M^{-1} cm^{-1}; TNP-Tyr, 13600 M^{-1} cm^{-1}; TNP-Gly, 12200 M^{-1} cm^{-1}. The substrates were prepared fresh before use and the blank reaction was followed similarly to that mentioned above. The incubation time was 2–4 h.

Hemoglobin or Casein. In a typical caseinase assay, 5 μL of crude extract was added to a buffer solution of 3.1 mL, containing 1.6% casein, and the mixture was incubated at pH 5.4 for 18 h at 30 °C. Two milliliters of a 10% TCA solution was then added to precipitate the proteins, and the absorbance of the peptides soluble in TCA was then measured at 276 nm (Tang, 1970; Reimerdes and Klostermeyer, 1976). The blank solution contained no enzyme extract during the incubation; the enzyme extract was added immediately before TCA precipitation. The optical density difference at 276 nm (1-cm cell path length) between the incubated and the blank solution typically was 0.08. Using 50 μL of crude extract, after an incubation time of only 5 h, ΔA^{1cm}_{276nm} was 0.26 (with a blank value of 0.42, most likely due in large part to polyphenolic compounds).

When hemoglobin was used as substrate, 5 μL of crude extract was added to a buffer solution (pH 4.0) of 3.1 mL containing 1.3% hemoglobin: ΔA^{1cm}_{276nm} was 0.56 after an incubation of 18 h. With 30 μL of crude extract and an incubation time of 4.5 h, the difference in absorbance was 0.27. The experiments reported in Figure 2 were performed with 5-h incubation times.

Azocoll or Hide Powder Azure. The assay with these insoluble substrates was carried out at 30–35 °C as described (Calbiochem-Behring Corp., 1979; Rinderknecht et al., 1968). The incubation was up to 24 h. In a typical assay, 0.1 mL of crude extract was added to 4 mL of a buffer solution containing 20 mg of azocoll or hide powder azure, and the mixture was incubated for 4 h. The difference in absorbance at 520 nm (1-cm cell path length) between the incubated solution and the blank was read at pH 8 (the region of highest activity) and was 0.08 for the azocoll and 0.04 at 595 nm for the hide powder azure assay.

Hexapeptide and B Chain of Insulin. The N-terminal hydrolysis of insulin B chain ($-SSO_3^-$) and the hexapeptide Leu-Trp-Met-Arg-Phe-Ala at pH 7.5 as catalyzed by the crude extract of ungerminated seeds was followed with thin-layer chromatography: after incubation of the polypeptides (1.7 and 3.2 mM, respectively) with a given amount of crude extract (39 or 195 μg of protein/100 μL of solution) at room temperature, the formation of free N-terminal amino acids was followed by separation on silica gel plates with phenol/phosphate buffer, 5 mM, pH 7.7, 75/25 (w/w) (Fahmy et al., 1961), after 2, 4, or 6 h of incubation. The amino acids were stained with ninhydrin and the R_f values were compared with those of standards. True carboxypeptidase activity—catalyzed by the crude extract of germinated seeds (5 days)—was followed with the hexapeptide at pH 5.0 in a way similar to that described above.

Milk-Clotting Activity. To obtain a reference characteristic of milk for protein and mineral content, a solution of 10% (w/v) of dry, defatted bovine milk with 1% $CaCl_2$ was prepared. The incubation time at 50 °C, between the addition of the enzyme and the first formation of milk clots, was used as a measure of the milk-clotting activity of the enzyme.

Chromatography, Electrophoresis, and Electrofocusing. The protein separation by gel permeation, ion-exchange, and affinity chromatography was followed at 280 nm with an Uvicord II UV detector (LKB 8300). For the molecular weight estimation of the eluted proteins, in the case of gel filtration, the column was calibrated with cytochrome *c* (13000), myoglobin (16900), ovalbumin (45000), and bovine serum albumin (68000). All the separations were carried out at 4 °C. Details are given in the figure legends. Purity tests of the partially purified aminopeptidase were made with a HPLC gel permeation

column (Synchropak GPC 100, Milton Roy Co. laboratory groups) by using Perkin-Elmer Series 2 pumps and a Perkin-Elmer LC 55/56 UV detector and recorder. The protein separations by SDS electrophoresis on polyacrylamide plates (7%) were carried out as described by Laemmli (1970) using a Bio-Rad cell, Model 220, for vertical gel slab electrophoresis thermostated at −10 °C and electrofocusing on Ampholine PAG plates (pH 3.5—9.5) using a Multiphor 2117 (LKB) system.

Spectrophotometers. All absorption measurements in the visible and UV range were carried out with an Uvikon 820 (Kontron) or a DB-GT (Beckman) spectrophotometer.

Reagents. The insulin B chain ($-SSO_3^-$) was prepared by sulfitolysis of insulin (porcine, Nordisk Insulin Laboratories) (Brandenburg, 1972) and separation of the two oxidized chains by ion-exchange chromatography on DEAE-cellulose at pH 3.7 (acetate buffer, 50 mM). The purity was tested by HPLC with a reversed-phase column (LiChrosorb RP 18 from Merck), using acetonitrile/phosphate buffer, pH 7.7, 5 mM, and $NaClO_4$, 0.1 M, as the eluant and with electrophoretic separation on cellulose acetate at pH 2.2 (2.4 M acetic acid with 7 M urea).

The hexapeptide Leu-Trp-Met-Arg-Phe-Ala, hemoglobin, Bz-DL-Lys-*p*-nitroanilide, phenylmethanesulfonyl fluoride, 4-(hydroxymercuri)benzoate, and tris(hydroxymethyl)aminomethane were purchased from Serva; the *p*-nitroanilides of Ala, Phe, Leu, and *N*-acetyl-Leu and Z-Phe-Ala, Z-Phe-Phe, L-Pro-β-naphthylamide, Ala-Gly, and Leu-Tyr were obtained from Bachem AG; Lys-*p*-nitroanilide, bovine serum albumin, Gly-*p*-nitroanilide, azocoll, hide powder azure, 2,4,6-trinitrobenzene-1-sulfonic acid (TNBS), and *p*-(hydroxymercuri)benzoate agarose were Sigma products; cytochrome *c*, myoglobin, ovalbumin, Glu-*p*-nitroanilide, Bz-DL-Arg-*p*-nitroanilide (BAPA), Coomassie Brilliant Blue R 250, ethylenediaminetetraacetic acid (EDTA), 2-mercaptoethanol, trichloroacetic acid (TCA), sodium tetraborate, and dimethyl sulfoxide (Me_2SO) were from Fluka; casein, Folin–Ciocalteus reagent, sodium disulfite, and sodium azide were from Merck; the protein assay was from Bio-Rad; Sephadex G-75 and G-100, carboxymethyl-Sephadex CM C-50, and diethylaminoethyl-Sepharose CL-6B were from Pharmacia; diethylaminoethyl (DEAE)-cellulose DE-52 was from Whatman.

RESULTS

Characterization of the Crude Extract of Ungerminated Seeds. First we ascertained the presence of aminopeptidases and carboxypeptidases in the crude extract by using the hexapeptide Leu-Trp-Met-Arg-Phe-Ala. Alanine and phenylalanine (see Figure 1a) were cleaved when the hexapeptide was incubated at pH 5 with a crude extract of seeds that had been germinated for 5 days (these extracts were used because the carboxypeptidase activity was high at the fifth day, as discussed later). When the hexapeptide was incubated with crude extracts of ungerminated seeds at pH 7.5, the first amino acid to be cleaved was leucine (Figure 1b), indicating the presence of aminopeptidases. This was also confirmed with similar experiments where the B chain of insulin (N-terminal amino acids phenylalanine and alanine) was used as the substrate.

Once the presence of "real" carboxy- and aminopeptidase was established, the activity was studied in greater detail by using substrates whose hydrolyses could be monitored spectroscopically and which are currently referred to in the literature (see Materials and Methods). For measuring the aminopeptidase activity, we tested Ala-*p*-nitroanilide, Lys-*p*-nitroanilide, Pro-β-naphthylamide, Phe-*p*-nitroanilide, Leu-*p*-nitroanilide, Glu-*p*-nitroanilide, Gly-*p*-nitroanilide, Leu-Tyr, and Ala-Gly. For the carboxypeptidase activity we used the two protected dipeptides Z-Phe-Ala and Z-Phe-Phe. Hemoglobin, casein, azocoll or hide powder azure, BAPA, Bz-DL-Lys-*p*-nitroanilide, and glutaryl-Phe-*p*-nitroanilide were used as substrates for endopeptidase activity. We studied the pH dependence of the hydrolysis catalyzed by the crude extract and we determined the optimal pH for each of the tested substrates. The pH profiles for the different substrates are shown in Figure 2. All the aminopeptidase substrates we used were readily hydrolyzed, with the exception of Glu-*p*-nitroanilide, which had only 5% of the activity of the other substates (this could be due to a particularly unfavorable K_m for this substrate). In agreement with literature data for aminopeptidases (Ryan and Walker-Simmons, 1981), the optimum pH is around 8 for all the tested substrates. Notice that the pH profiles are very similar for the first five substrates (Figure 2a–e), whereas Leu-Tyr and Pro-β-naphthylamide have slightly different behaviors. (The pH profile depends not only on the nature of the ionizable groups of the enzyme's active site but also on the ionization behavior of the substrate). The fact that the hydrolysis of Phe-*p*-nitroanilide and Leu-*p*-nitroanilide (parts e and d of Figure 2) is due to exopeptidases (in contrast to endopeptidases) is substantiated by the lack of activity found for the two corresponding N-protected compounds, Ac-Leu-*p*-nitroanilide and glutaryl-Phe-*p*-nitroanilide (used at concentrations of 0.3 and 0.2 mM, respectively). The last data rule out the existence of α-chymotrypsin-like activity. This last finding also demonstrates that the aminopeptidase activity is not due to the action of nonspecific endoarylamidases. It could still be due to an exoarylamidase (Bergmeyer, 1970). In this paper, we will use the generic term "aminopeptidase" without further reference to this question.

The two protected dipeptides used as substrates for carboxypeptidase activity were hydrolyzed at pH optimum 5, as shown in Figure 2h, i.

In the literature there are reports that casein (Schnarrenberger et al., 1972) and hemoglobin (Goldovskii and Mirzoev, 1979; Lorenz, 1980) are substrates for sunflower seed extracts. In our case, there were considerable experimental difficulties in clarifying this point: the polyphenolic compounds present in the meal interfere with the

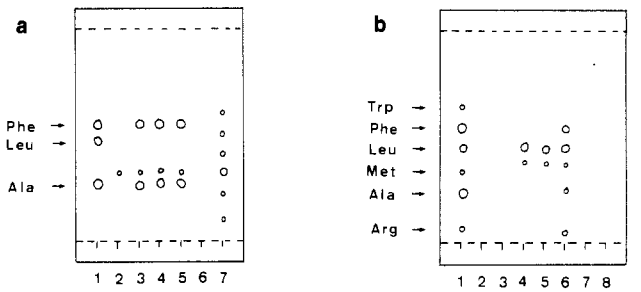

Figure 1. Test of exopeptidase activity. Incubation of Leu-Trp-Met-Arg-Phe-Ala with crude extracts of ungerminated seeds (b) or of seeds germinated for 5 days (a) at pH 7.5 (b) or pH 5.0 (a). Thin-layer chromatographic separation of the amino acids after the incubation [silica gel plates; phenol/phosphate buffer, pH 7.5, 0.05 M, 75/25 (w/w); detection with ninhydrin]. (a) Carboxypeptidase test, pH 5.0: 1, amino acid standards; 2, after an incubation of 15 min; 3, after an incubation of 90 min; 4, after an incubation of 150 min; 5, after an incubation of 210 min; 6, Leu-Trp-Met-Arg-Phe-Ala; 7, crude extract of seeds germinated for 5 days. (b) Aminopeptidase test, pH 7.5: 1, amino acid standards; 2, after an incubation of 5 min; 3, after an incubation of 15 min; 4, after an incubation of 30 min; 5, after an incubation of 35 min; 6, after an incubation of 360 min; 7, Leu-Trp-Met-Arg-Phe-Ala; 8, crude extract of ungerminated seeds.

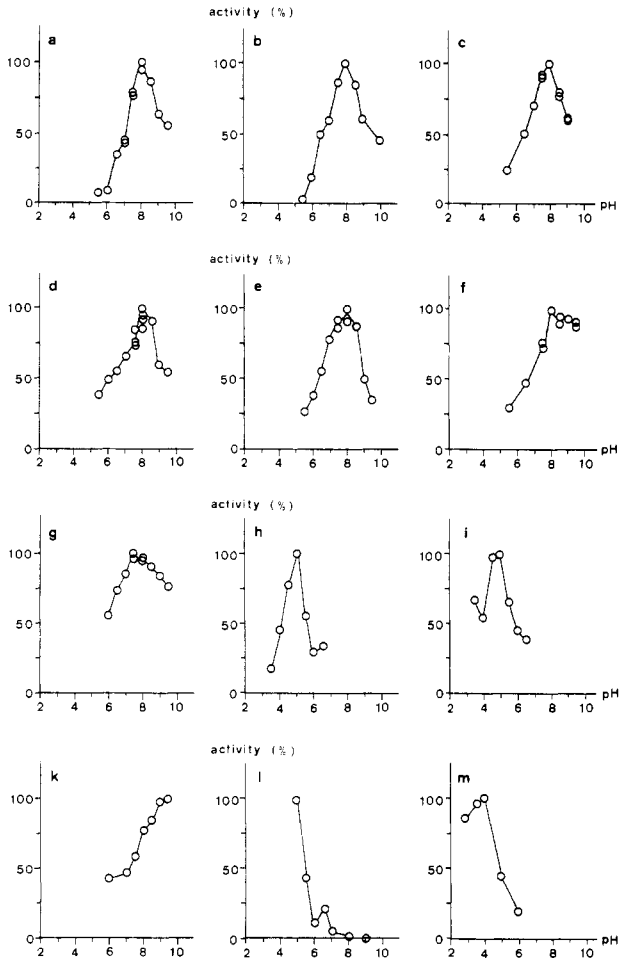

Figure 2. Relative proteolytic activities of the crude extracts, pH dependence. For each substrate, the activity was defined as 100% at the optimum pH. (a) Lys-*p*-nitroanilide, 0.8 mM (22.8); (b) Ala-*p*-nitroanilide, 0.9 mM (26.3); (c) Ala-Gly, 3.8 mM (16.9); (d) Leu-*p*-nitroanilide, 0.9 mM (11.0); (e) Phe-*p*-nitroanilide, 0.3 mM (11.9); (f) Leu-Tyr, 3.8 mM (44.8); (g) Pro-β-naphthylamide, 0.9 mM (18.7); (h) Z-Phe-Phe, 0.4 mM (23.9); (j) Z-Phe-Ala, 1.0 mM (10.9); (k) Bz-DL-Arg-*p*-nitroanilide, 0.8 mM (2.8); (l) casein, 1.6 wt %; (m) hemoglobin, 1.3 wt %. The specific activity at the optimum pH (μmol of substrate·min^{-1}) (mg of crude extract protein)$^{-1}$; reaction volume, 3 mL; T = 30 °C) is in parentheses.

reading at 276 nm (used to determine the concentration of the TCA-soluble peptides); the proteins of the crude extracts of sunflower meal are not so soluble at the low pHs required for the casein and hemoglobin assays. Due to this, our assays required longer incubation times, and the ΔA readings had to be made against high absolute absorbances. Despite this, the presence of a protease (casein and hemoglobin as substrates) in our extract appears certain. The catalyzed hydrolysis of hemoglobin and of casein is maximal in the acid pH range. the pH optimum with hemoglobin as the substrate is around 4 (Figure 2m), while with casein, it could not be determined (see Figure 2l), due to substrate insolubility below pH 4. No significant activity was found with azocoll and hide powder azure substrates.

To determine the minimal number of enzymes that is responsible for the catalytic hydrolysis of the tested substrates, we separated the proteins of the crude extract by gel filtration. The chromatograms and the activity profiles for a number of the substrates are shown in Figure 3. The first six activity profiles are diagnostic for aminopeptidase activity. From the analysis of the chromatographic pattern we can conclude that at least three different aminopeptidases must be present in the crude water extract. Figure 3h shows that there must be at least one enzyme

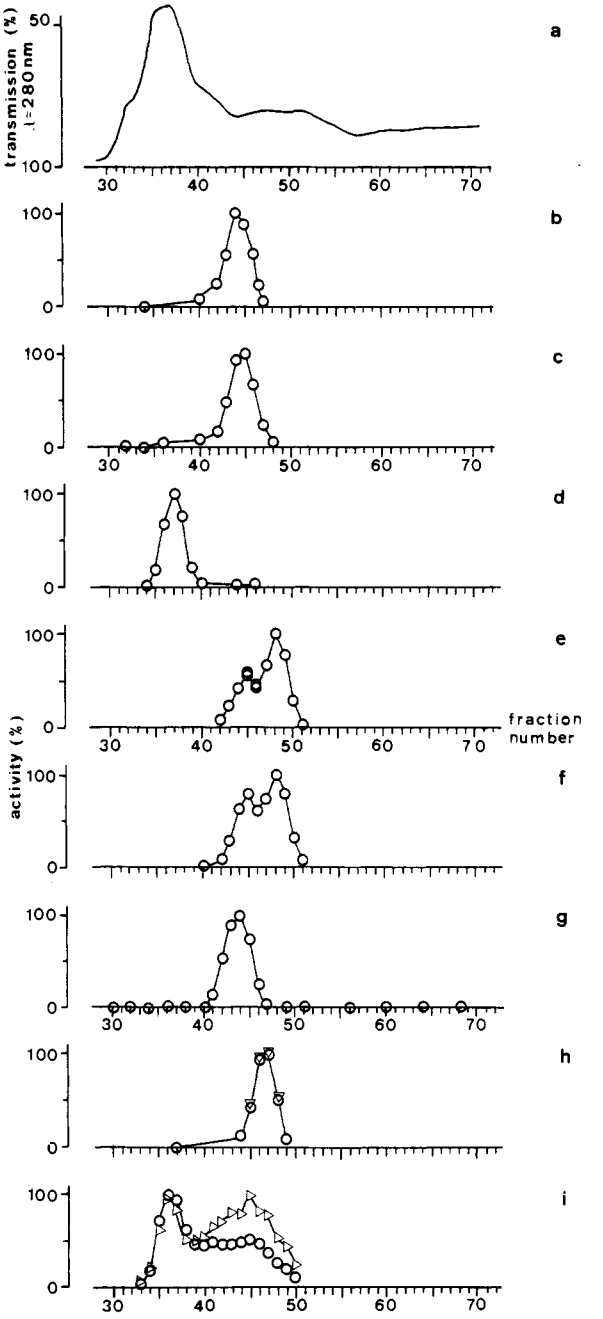

Figure 3. Gel filtration of the crude extract. (gel, Sephadex G-100 sF; column, 2.5 cm × 95 cm; flow rate, 4.7 mL/h; 1 h/fraction; elution buffer, phosphate, 50 mM, pH 7.5, and 0.02% NaN_3; sample volume, 8.5 mL). (a) Transmission at 280 nm. (b–i) Activity against different substrates: (b) Ala-*p*-nitroanilide (pH 7.5); (c) Lys-*p*-nitroanilide (pH 7.5); (d) Pro-β-naphthylamide (pH 7.5); (e) Phe-*p*-nitroanilide (pH 7.5); (f) Leu-*p*-nitroanilide (pH 7.5); (g) Ala-Gly (pH 8.0); (h) Bz-DL-Arg-*p*-nitroanilide [(○) pH 8.6] and Bz-DL-Lys-*p*-nitroanilide [(△) pH 8.6]; (i) Z-Phe-Ala [(○) pH 5.0] and Z-Phe-Phe [(△) pH 5.0].

with trypsin-like activity, whereas the last figure (Figure 3i) indicates the presence of at least two enzymes with carboxypeptidase activity.

As a first step for the characterization of the proteases found in sunflower meal, we attempted to purify the aminopeptidase that acts upon Lys-*p*-nitroanilide. The classical methods of protein purification, such as ion-exchange chromatography and gel filtration, provided a fraction that was homogeneous by HPLC (gel filtration). The molecular weight was around 65 000 as determined by gel filtration (Sephadex G-75 and G-100) and the optimum pH for the hydrolysis of Lys-*p*-nitroanilide was

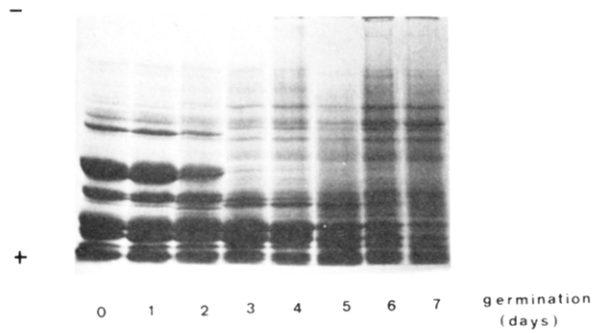

Figure 4. SDS electrophoresis on polyacrylamide gels (7%) of soluble proteins during germination.

found to be 7.2. A more rigorous analysis of the purity of this fraction by analytical isoelectric focusing on PAGE showed some heterogeneity; furthermore, activity tests showed partial contamination with other activities (e.g., BAPA).

In order to find a specific inhibitor for this partially purified aminopeptidase, several compounds with potential protease inhibition activities were investigated. Phenylmethanesulfonyl fluoride (<7 mM)—as a typical inhibitor for serine proteases—and EDTA (<4 mM)—as an inhibitor for metalloproteases—were ineffective. With inhibitors for SH proteases, *p*-(hydroxymercuri)benzoate, or Hg^{2+}, the activity was reduced to about 5% of the original activity by using 3 mM inhibitor solution (protein concentration, 34 μg/mL; substrate concentration, 0.8 mM, pH 7.2).

A purification of the aminopeptidase by affinity chromatography with *p*-(hydroxymercuri)benzoate agarose was not successful, probably due to the unspecific binding of other SH-containing proteins. Work is now in progress to achieve a better purification.

Evolution of Soluble Seed Proteins and Proteolytic Activity during Germination. One of the main goals in our research is to clarify how the protein composition and the activity of the enzymes in defatted sunflower meal change during germination. This paper presents the first series of data in regard to sunflower. The amount of water-soluble proteins in defatted meal of germinated seeds is maximal around the second and third day of germination. Specifically, under our experimental conditions, we obtained 3.9 mg of protein/mL of extract from meal of ungerminated seeds, from 1-day germination 5.8 mg/mL, from 2 days 9.1 mg/mL, from 3 days 10.5 mg/mL, from 5 days 4.4 mg/mL, and from 6 days 4.5 mg/mL. Figure 4 shows the SDS–PAGE pattern of the soluble proteins of germinating seeds. In the protein profile of the extract of ungerminated seeds, six main bands are present, which do not significantly change during the first 2 days of germination. After that, dramatic changes in the protein pattern occur: some of the very intense middle bands disappear and new, high molecular weight polypeptides are detected.

Similar features of the disappearance of proteins and the formation of new ones during germination are also observable with ion-exchange chromatography (Figure 5). The last peak in the elution pattern of Figure 5a, which is largely due to polyphenolic compounds, is dramatically decreased; as already reported (Ruckenbrod, 1955), these compounds are metabolized during germination. We have not yet studied the details of the protease activity in such elution fractions. Preliminary data indicate the presence of at least two aminopeptidases that catalyze the hydrolysis of Phe-*p*-nitroanilide. This is in agreement with the observation obtained from gel filtration (see Figure 3e). The

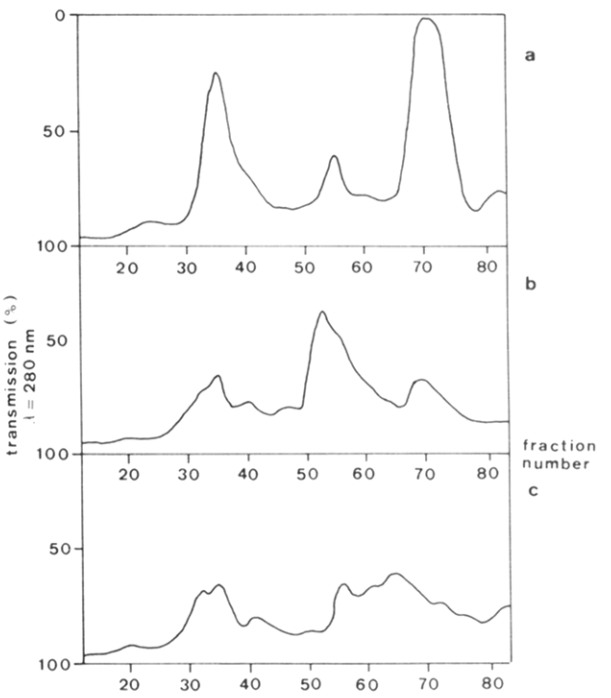

Figure 5. Ion-exchange chromatography of crude extracts during germination. (gel, DEAE-Sepharose CL-6B; column, 3 cm × 26 cm; flow rate, 23 mL/h; 12 min/fraction; elution buffer, phosphate, 50 mM, pH 7.5, and 0.02 NaN_3; 0 M NaCl → 0.5 M NaCl, linear gradient; sample volume, 8.5 mL). (a) Extract of ungerminated seeds; (b) extract of germinated seeds for 3 days; (c) extract of germinated seeds for 5 days.

enzyme that catalyzes the hydrolysis of Pro-β-naphthylamide elutes practically together with the above-mentioned aminopeptidases. That these are really different enzymes was evidenced by gel filtration (see Figure 3d,e), as the BAPAase elutes in the ion-exchange chromatographic pattern earlier than the above-mentioned aminopeptidases.

The protease activity in extracts of seeds during germination was followed with Phe-*p*-nitroanilide, Leu-*p*-nitroanilide, Ala-*p*-nitroanilide, Lys-*p*-nitroanilide, Pro-β-naphthylamide, Z-Phe-Ala, BAPA, glutaryl-Phe-*p*-nitroanilide, casein, and hemoglobin. No marked changes in aminopeptidase activity (pH 7.5) could be observed with Leu-*p*-nitroanilide and Phe-*p*-nitroanilide, and also in the case of Pro-β-naphthylamide changes were within 20%. For Ala-*p*-nitroanilide (pH 7.5) and Lys-*p*-nitroanilide (pH 7.5), the activity decreases in the later stage of germination and reaches about 30–40% of the initial value after 6 days (see parts a and b of Figure 6); a similar behavior was shown in the case of hemoglobin and casein (data not shown), where also the activities reached ca. half of the initial value after 6 days of germination.

The greatest activity changes observed were for the hydrolysis of Z-Phe-Ala at pH 5.0 (Figure 6c) and BAPA at pH 8.6 (Figure 6d): during the first 2–3 days there were no significant activity changes; afterward there was a marked increase (250–500% compared to the activity of ungerminated seeds).

As with the extracts of ungerminated seeds, during germination there was no detectable activity with the chymotrypsin substrate glutaryl-Phe-*p*-nitroanilide.

Milk Clotting Activity of Defatted Meal of Sunflower Seeds. We have observed (Walde et al., 1982) that defatted sunflower meal or water extracts of it are capable to clot bovine cow's milk. Clotting is more readily obtained if $CaCl_2$ is added to the milk to a final concentation of ca. 1% with an incubation temperature of 50 °C; Figure 7 shows the clotting time as a function of the amount of meal

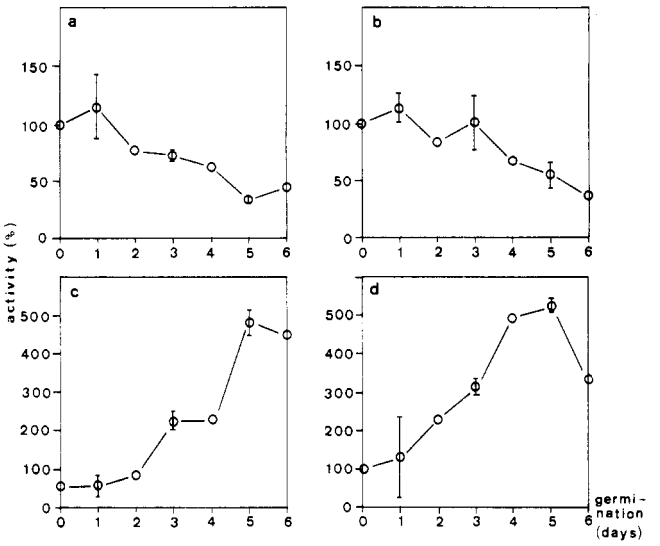

Figure 6. Changes in proteolytic activities of crude extracts of germinating seeds, as measured with different substrates: (a) Ala-*p*-nitroanilide, 0.9 mM (pH 7.5); (b) Lys-*p*-nitroanilide, 0.8 mM (pH 7.5); (c) Z-Phe-Ala, 1.0 mM (pH 5.0); (d) Bz-DL-Arg-*p*-nitroanilide, 0.8 mM (pH 8.6). The bar represents the reproducibility when different germination batches are used.

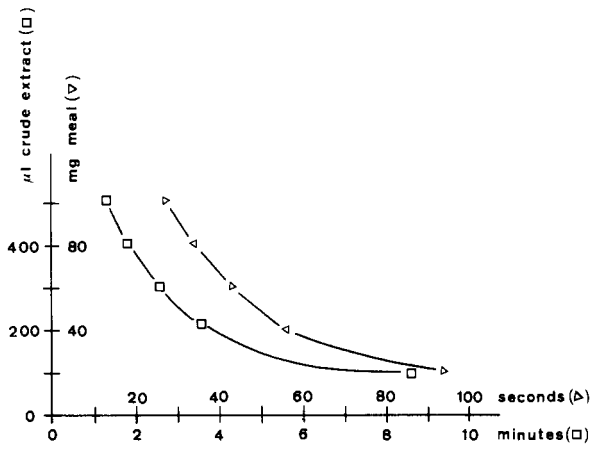

Figure 7. Bovine milk clotting activity of defatted sunflower meal and its crude extract: meal (△) or crude extract (□) added to 3 mL of defatted milk (10%) vs. clotting time. $CaCl_2$ concentration, 1 wt %; T = 50 °C; ungerminated seeds.

of ungerminated seeds and as a function of crude water extract added (see Materials and Methods). A very efficient clotting is obtained at concentrations of added defatted meal as low as 1%. As expected, when the temperature is decreased and the pH is increased, the clotting time increases. For example on going from pH 5 to pH 6 it increases by a factor of ca. 5. The concentration of $CaCl_2$ has also a marked effect, with an optimal value of 0.7% at pH 6.2. This optimal concentration of $CaCl_2$ decreases by decreasing pH.

This finding may be of significance for cheese production, particularly when no contamination with animal rennet or other animal sources is desired. Furthermore, rennet is becoming more and more expensive, and alternative enzymatic preparations can be of biotechnological importance. Microbial preparations have been already investigated and utilized (Green, 1977). It is also interesting to mention that other plant extracts seem to have the same property as sunflower meal (Vieira de Sá and Barbosa, 1972; Barbosa et al., 1981; Tavasolian and Shabbak, 1979).

We have attempted some preliminary experiments in order to see whether sunflower meal can be used for cheese production. Typically, a fresh precipitate, with a pleasant, neutral taste and no unpleasant odor, is obtained. However, in our first tests the cheese developed a bitter taste on standing. Experiments are in progress for improving the quality of the preparations.

DISCUSSION

Protease activity (using casein and hemoglobin as substrates) at acidic pH and BAPAase activities were found in extracts of sunflower seeds (Schnarrenberger et al., 1972; Goldovskii and Mirzoev, 1979; Lorenz, 1980) as well as in extracts of a number of other seeds [for example, Salmia (1981), Beevers (1968), Tully and Beevers (1978), Chrispeels and Boulter (1975), Graf and Hoagland (1969), Hoagland and Graf (1974), Grange et al. (1980), Harris and Chrispeels (1975), Burger et al. (1968) Cameron and Mazelis (1971), Dunaevskii et al. (1976), Emtseva and Belozerskii (1977), Yatsu and Jacks (1967), and St. Angelo et al. (1969a,b, 1970)].

Sunflower seeds seem to contain at least two types of endopeptidases, one working in the acid pH range (casein, hemoglobin, and milk-clotting activity) and one catalyzing the hydrolysis of BAPA at alkaline pH. This is also indicated by the different activity behaviors during germination: a general decrease in the protease activity toward casein and hemoglobin and an increase in the BAPAase activity. This picture also holds for other plant seeds. For example, in the case of mung bean cotyledons [*V. radiata* (L.) Wilczek] it has been shown that the BAPAase activity is not identical with the activity of the vicilin peptidohydrolase, the endopeptidase that seems to be responsible for the starting of the protein degradation during germination (Baumgartner and Chrispeels, 1977).

As in other seeds—for example, rape (*Brassica napus* L.) (Barth and Hermann, 1974), pea (*Pisum sativum* L.) (Elleman, 1974), barley (*Hordeum vulgare* L.) (Mikola, 1978), and Scots pine (*Pinus sylvestris* L.) (Salmia, 1981)—in sunflower seeds several aminopeptidases, at least three, could be identified. They can be described as follows: A-1, a proline iminopeptidase that catalyzes the hydrolysis of Pro-β-naphthylamide (activity maximum in fraction 37 of Figure 3); A-2, an enzyme that catalyzes the hydrolysis of Ala-*p*-nitroanilide, Ala-Gly, Lys-*p*-nitroanilide, Leu-*p*-nitroanilide, or Phe-*p*-nitroanilide (activity maximum in fraction 44/45 of Figure 3); A-3, an enzyme that catalyzes the hydrolysis of Phe-*p*-nitroanilide or Leu-*p*-nitroanilide (activity maximum in fraction 48 of Figure 3). The molecular weights of all the sunflower seed aminopeptidases are in the range of 50 000–90 000, similar to those of the aminopeptidases in other seeds, e.g., pea (*P. sativum* L.) 58 000 and 74 000 (Elleman, 1974), rapeseed (*Brassica napus* L.) 79 000 (Barth and Hermann, 1974), and barley (*H. vulgare* L.) 65 000 (Kolehmainen and Mikola, 1971). As far as specificity is concerned, these three enzymes, A-1, A-2, and A-3, appear to be very similar to the pea seed aminopeptidase described by Elleman in 1974, who tested the enzymes by specific staining after electrophoretic separation.

Our data indicate that there are at least two carboxypeptidases in ungerminated sunflower seeds. Carboxypeptidases in plant seeds have been reported several times in literature and have been clearly ascertained, at least in the cases of barley (*H. vulgare* L.)—substrate Z-Phe-Ala, molecular weight 90 000 (Visuri et al., 1969)—and soybeans [*Glycine max.* (L.) Merr.]—substrate Z-Glu-Phe, molecular weight 99 000 and 106 000 (Kubota et al., 1976).

A dipeptidase similar to the one found in barley (*H. vulgare* L.)—substrate Ala-Gly, molecular weight 130 000–175 000 (Sopanen, 1976)—with a higher molecular

weight than other proteases was not found in sunflower seeds. As it is clear from Figure 3, the hydrolysis of Ala-Gly is catalyzed by the same enzyme that catalyzes the hydrolysis of Ala-*p*-nitroanilide, i.e., the aminopeptidase A-2.

In conclusion, it is clear from our data that there is at least qualitatively a great similarity between the proteolytic activity of enzymes in sunflower seeds and enzymes in other seeds (angiosperms and gymnosperms). Figure 4 demonstrates that dramatic changes in the soluble protein pattern occur on the third day of germination, which correlates with an increase in carboxypeptidase activity. Particularly interesting is the case of this carboxypeptidase activity, with its drastic increase on the fifth–sixth day. This is attended by an analogous pattern for the BAPAase activity. Inasmuch as this last activity corresponds to an endopeptidase activity, one may propose that both enzymes (endopeptidase and carboxypeptidase) are strongly involved in the degradation of the storage protein, i.e., helianthinin. However, our "alkaline" BAPAase activity cannot be interpreted straightforwardly as a reserve protein degrading endopeptidase, since its activity is displayed in an unusual alkaline pH range. Actually, the picture concerning the endopeptidases in sunflower seeds is still unclear (remember at this regard also the lack of activity against azocoll and hide powder azure). An increase in carboxypeptidase activity—together with an increase in protease activity (casein as the substrate)—was also detected in the cotyledons of germinating mung beans [*V. radiata* (L.) Wilczek] (Chrispeels and Boulter, 1975) and in germinating peanuts (*A. hypogaea* L.) (Mikola, 1976).

The changes in aminopeptidase activity during germination are not so marked; therefore, it is even more difficult to provide a rationale for them. One should point out that with pea seeds (*Pisum sativum* L.), the aminopeptidase activity was not specifically connected to the mobilization of the storage protein but connected to the general turnover of the cellular proteins (Collier and Murray, 1979).

Another important question arising from the data of Figure 6 is if and to what extent protease inhibitors are involved in the changes in activities. For example, the apparent discrepancy between our data and literature data about the time course of the protease activity (casein as the substrate) can be interpreted in this respect. In fact, we have found that the protease activity (hemoglobin and casein as substrates) decreases, whereas Schnarrenberger et al. (1972), who studied the protease activity (using casein as the substrate) of isolated protein bodies from germinating sunflower cotyledons, found an increase in activity. Our data, which are for a protease of the whole seedling, may be explained by assuming that a strong proteinase inhibitor, formed during germination, is localized outside the protein bodies, possibly in the cytoplasm. Indeed, in the case of mung bean cotyledons [*V. radiata* (L.) Wilczek] (Baumgartner and Chrispeels, 1976; Chrispeels and Baumgartner, 1978), an inhibitor of the vicilin peptidohydrolase and one of the exogenous endopeptidases, trypsin, was found in this part of the cell. In general, this observation leads to the question of the presence of protease inhibitors in sunflower meal. In the literature there are indications to the existence of weak trypsin inhibitor activity (Agren and Liedén, 1968; Roy and Bhat, 1974). However, the presence of specific inhibitors for endogenous sunflower proteases has never been reported. We are further investigating this point.

Finally, along quite a different line of interest, one should mention the possible biotechnological importance of the milk-clotting activity of the very cheap, readily available sunflower meal. This property certainly adds to the broad chemical and biochemical interest of sunflower seed proteins.

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Registry No. Proline iminopeptidase, 9025-40-5; carboxypeptidase, 9031-98-5; aminopeptidase, 9031-94-1; endopeptidase, 9001-92-7; exopeptidase, 9031-96-3.

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Multiple Discriminant Analysis in the Analytical Differentiation of Venetian White Wines. 4. Application to Several Vintage Years and Comparison with the *k* Nearest-Neighbor Classification

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Fifty-three samples of the Venetian white wines Soave Classico, Prosecco di Conegliano-Valdobbiadene, and Verduzzo del Piave, collected from vintages of 1980 and 1981, were analyzed for the parameters considered in previous papers. Results confirm previous findings on the variability of products through the years. Multiple discriminant analysis was applied to all the available data in our file [i.e., referred to samples of 1977, 1980, and 1981 Soave, 1977, 1979, and 1980 Prosecco, and 1977 and 1981 Verduzzo wines]. The apparent error rate was about 12.3%. The expected actual error rate was estimated both by the jackknife procedure and the learning set/test set partition method. Results were almost coincident (about 18%). A definite overlap between the Prosecco and the Verduzzo areas in the discriminant space was revealed. Results obtained by the *k* nearest-neighbor classification show that the two methods of pattern recognition have, in this case, practically the same classification power.

Previously, we reported on the applicability of the statistical method of multiple discriminant analysis to the problem of differentiation of three Venetian white wines (Soave Classico, Prosecco di Conegliano-Valdobbiadene, and Verduzzo del Piave) by means of the following analytical parameters: sodium, potassium, calcium, magnesium, chloride, pH, titratable acidity (TA), phosphorus, ash content (AC), and alkalinity of the ashes (AA) (Moret et al., 1980; Scarponi et al., 1981). Also, it was shown that, owing to the possible high variability of the products through the years, data from several vintage years need to be processed in order to obtain conclusive results (Scarponi et al., 1982).

Continuing this program, in this paper we report the results of the analytical measurements mentioned above and performed on samples obtained from 1980 and 1981 vintage years. The multiple discriminant analysis was repeated by using all the data, and the classification results, together with the estimate of the expected actual error rate, are reported. Moreover, the data set was processed by the reference, nonparametric classification rule of the *k* nearest neighbors (kNN). Results obtained by the two pattern recognition methods are compared.

EXPERIMENTAL SECTION

Collection and Analysis of Samples. Eight and ten samples of wine Soave, vintage of 1980 and 1981, respec-

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