Partial Purification and Characterization of a Proteolytic Activity of Alfalfa Juice

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The proteolytic activity of alfalfa (*Medicago sativa*) press juice has been partially purified by a combination of ammonium sulfate precipitation, gel filtration on Sephadex G-100 and Sephadex G-200, and ion-exchange chromatography on DEAE-cellulose. The enzyme shows a pH optimum between 7 and 9. The optimum temperature is close to 45 °C. The activity is strongly inhibited by acetylleucylleucylargininal (Leupeptin). The enzyme is insensitive to chelating agents and to sulfhydryl group reagents. This protease probably accounts for the disappearance of some proteins from alfalfa juice during storage.

The decay of enzyme levels in alfalfa (*Medicago sativa*) extracts stored at 37 °C for different time intervals was reported in a previous paper (Camici et al., 1980). These findings were ascribed to proteolytic activities shown to be present in alfalfa by Singh (1965), de Fremery et al. (1972), and Pirie (1978). In this paper we describe the partial purification of a neutral protease and report some of its general properties.

EXPERIMENTAL SECTION

Materials. Sephadex G-100 and Sephadex G-200 were obtained from Pharmacia (Uppsala, Sweden); DEAE-cellulose, nominal capacity 0.73 mequiv min^{-1} g^{-1}, was obtained from Serva Feinbiochemia (Heidelberg, Germany); N^{α}-benzoyl-DL-arginine-*p*-nitroanilide (DL-BAPNA), phenylmethane sulfonyl fluoride (PMSF), *p*-nitrophenyl *p'*-guanidinobenzoate hydrochloride (*p*-NPGB), *p*-(chloromercuri)benzoic acid (PCMB), and ethylendiaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA), casein, and other chemicals reagents were obtained from Merck (Darmstadt, Germany). The synthetic tripeptide acetylleucylleucylargininal (Leupeptin) was a kind gift of Dr. G. Borin of the Centro di Studi sui Biopolimeri, C.N.R. (Padova, Italy).

Enzyme Purification. Unless otherwise specified, all the operations were carried out at 4 °C. Large volumes of press juice with low protein concentration (1.3 mg/mL) have to be processed in order to prepare sufficient amounts of partially purified enzyme for physicochemical studies. Since preliminary observations have shown no decrease of activity after lyophilization of crude extracts, we used lyophilization as the first step with the aims of reducing the volumes to a more suitable size with larger protein concentration and providing us with a large amount of starting material to be used for subsequent preparations throughout long periods of time. The purification procedure is summarized in Table I. Fifty grams of lyophilized material obtained from press juice prepared according to Camici et al. (1980) was resuspended in 37 mL of 0.05 M Tris-HCl buffer, pH 8 (crude extract). The suspension was brought to 50% saturation with ammonium sulfate by the slow addition of a saturated buffered solution of the salt. The resultant suspension was allowed to stand for 3 h at 4 °C and was then centrifuged at 15000*g* for 30 min. The pellet, which contained the entire proteolytic activity, was resuspended in 15 mL of the above buffer. The resulting yellow solution was applied to a column (2.3 × 122 cm) of Sephadex G-100 equilibrated with 0.05 M Tris-HCl buffer, pH 8. The protease is eluted immediately after the major protein peak, and a large portion of the yellow color was retarded on the column. The active fractions were pooled. The pooled fractions were brought to 80% saturation with ammonium sulfate, by the slow addition of the solid salt. The resultant suspension was allowed to stand for 3 h at 4 °C and was then centrifuged at 15000*g* for 30 min. The pellet was dissolved in 3.3 mL of the same buffer. The solution was then applied to a column (2 × 65 cm) of Sephadex G-200 equilibrated with 0.05 M Tris-HCl buffer, pH 8. The enzyme was eluted as a sharp peak of activity. The active fractions were pooled and applied to a column (1.6 × 21 cm) of DEAE-cellulose equilibrated with 0.05 M Tris-HCl buffer, pH 8. The initial peak of protein was washed through with the same buffer, and the column was then eluted by a linear gradient of 0–0.8 M NaCl in 0.05 M Tris–acetate buffer, pH 6 (Figure 1). The fractions containing the enzyme activity (0.25–0.35 M NaCl) were pooled, immediately brought to pH 8, and stored at 2 °C.

Table I. Purification of Alfalfa Neutral Protease

step	volume, mL	units[a]	protein, mg	units/mg	yield units, %
crude extract	37	28.5	648	0.04	100
0–50% $(NH_4)_2SO_4$	15	28.5	343	0.08	100
Sephadex G-100	50	17.5	50	0.35	61.4
0–80% $(NH_4)_2SO_4$	3.3	12.2	30	0.41	42.8
Sephadex G-200	31.5	11.6	25	0.46	40.9
DEAE-cellulose	13.5	1.9	0.9	2.11	6.6

[a] One enzyme unit is the amount of enzyme catalyzing the transformation of 1 μmol of substrate/min.

Enzyme Assay. The activity of purified protease was assayed by using casein as the substrate according to Kunitz (1947). The assay mixture was as follows: 0.5 mL of enzyme solution, 1.0 mL of 0.1 M Tris-HCl buffer, pH 8, and 1.0 mL of casein solution (10 mg/mL) dissolved in the same buffer. At alkaline pH the purified alfalfa protease is active on casein at both 37 and 40 °C, and the activity is linear with time up to 120 min under both conditions (Figure 2). The routine assays for the purification steps and the general characterization reported in this paper were carried out by using DL-BAPNA as the substrate. The spectrophotometric assay was carried out at 37 °C by measuring the optical density increase at 405 nm, according to Erlanger et al. (1961). The assay mixture was as follows: 0.1 M Tris-HCl buffer, pH 8, 2.2 mM DL-BAPNA, and various amounts of enzyme solution in a final volume of 1 mL.

Protein Determination. Protein content was estimated by the method of Lowry et al. (1951), using BSA as the standard, and by the optical densities at 280 and 260 nm according to Layne (1957).

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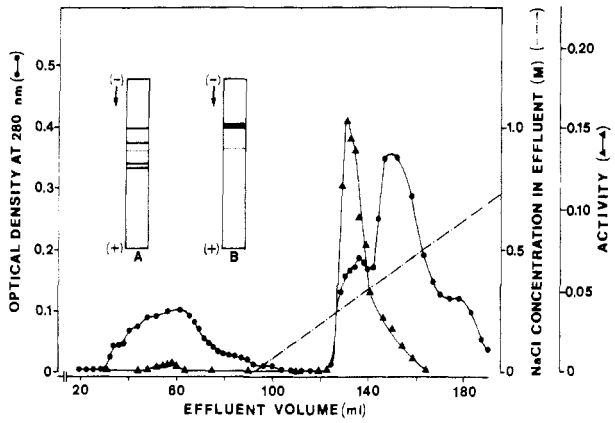

Figure 1. Chromatography of alfalfa protease on a DEAE-cellulose column. Experimental conditions are given in the text. Activity is expressed as micromoles of substrate transformed per minute per milliliter at 37 °C. The inset diagram shows the acrylamide electrophoretic pattern of the (A) Sephadex G-200 preparation applied on the DEAE-cellulose column and (B) DEAE-cellulose pool.

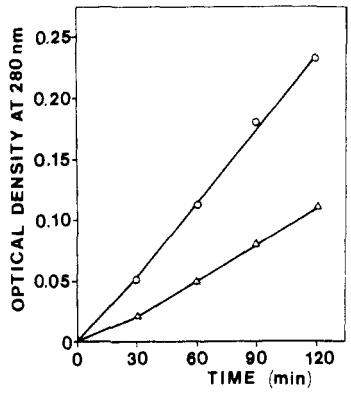

Figure 2. Hydrolysis rates of casein by the purified protease, at 37 °C (△) and 40 °C (○).

Molecular Weight. This determination was carried out according to Andrews (1965) by gel filtration on a Sephadex G-100 column (1.2 × 80 cm), equilibrated with 0.05 M Tris-HCl buffer, pH 8. A mixture of protease preparation and marker proteins of known molecular weight was applied to the top of the column, which was then eluted with the same buffer, at a flow rate of ~9 mL/h. The void volume for myoglobin (molecular weight 17 000) was estimated by absorption at 407 nm; the void volumes for BSA monomer (molecular weight 68 000) and BSA dimer (molecular weight 136 000) were estimated by absorption at 280 nm; the void volumes for adenosine deaminase from calf intenstinal mucosa (molecular weight 34 000) and adenosine deaminase from *Bacillus cereus*, prepared according to Serra et al. (1971) (molecular weight 77 000), were estimated by the method of Kalckar (1947). The protease was located by assaying the effluent as described before. The effluent volume is plotted against the logarithm of the molecular weight (Figure 3).

Disc Gel Electrophoresis. Disc gel electrophoresis was carried out at pH 9 using a 7.5% acrylamide system as described by Davis (1964).

RESULTS AND DISCUSSION

The effect of pH on both the stability and the activity of the purified proteolytic enzyme is reported in Figure 4. The proteolytic activity is stable at pH values over 5 after storage overnight at 4 °C; the stability decreases rapidly at pH values lower than 5 (Figure 4A). This finding is in accordance with our previous observations that

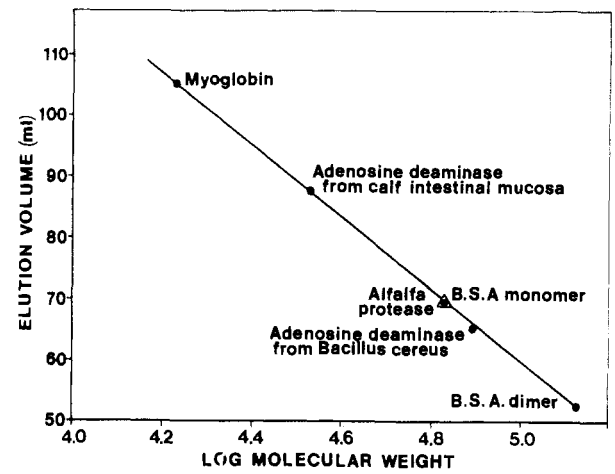

Figure 3. Plot of elution volume against log molecular weight for alfalfa protease and other marker proteins on Sephadex G-100.

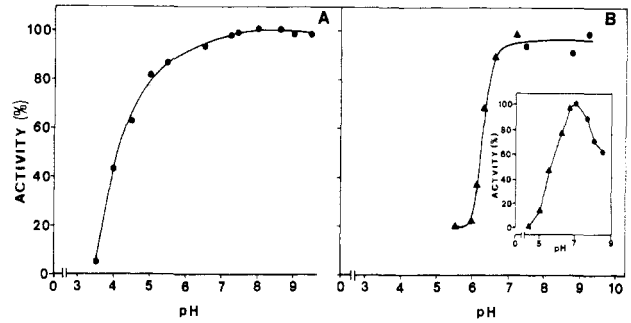

Figure 4. (A) Effect of storage overnight at 40 °C at different hydrogen ion concentrations on the protease activity. Portions of the final enzyme preparation were held at the indicated pH values and then assayed in Tris-HCl buffer at pH 8. (B) Effect of hydrogen ion concentration on the protease activity of the final preparation. Enzyme activities were assayed in Tris–acetate buffer (▲) and in Tris-HCl buffer (●). In the inset, effect of the hydrogen ion concentration on the protease activity of the crude extract of alfalfa, prepared according to Camici et al. (1980), is reported. Enzyme activities were assayed in Tris–acetate buffer (▲) and Tris-HCl buffer (●).

higher yields of protein isolates are obtained when the alfalfa juice is processed at acid pH (Camici et al., 1980).

The optimum activity is reached at pH 6.5 and remains constant at least up to pH 9 (Figure 4B), as shown for other proteolytic activities (Laskowski et al., 1966). The results reported in the inset of Figure 4B showing that the proteolytic activity of crude press juice rapidly falls at pH values higher than 7 might be ascribed to several factors, including the presence of inhibitors acting at alkaline pH values (Chang et al., 1978; Sosulski et al., 1972; Bittoni et al., 1977).

Samples of the purified enzyme stored at −20 °C overnight become completely inactive. However, it is stable between 4 and 45 °C; 100% inactivation occurs at 60 °C (Figure 5A). Figure 5B shows that maximal activity is observed at 45 °C. Similar findings have been reported for several proteolytic activities from vegetal sources (Drivdahl and Thimann, 1977; Harvey and Oaks, 1974; Hashinaga et al., 1978).

The molecular weight of the alfalfa protease is 68 000 as measured with the Andrews (1965) method and favorably compares with the molecular weight (76 000) of the neutral protease of oat leaves determined by Drivdahl and Thimann (1977).

The proteolytic activity is insensitive to a large number of cations, to chelating agents, and to reduced and oxidized sulfhydryl group reagents, whereas it is sensitive to low

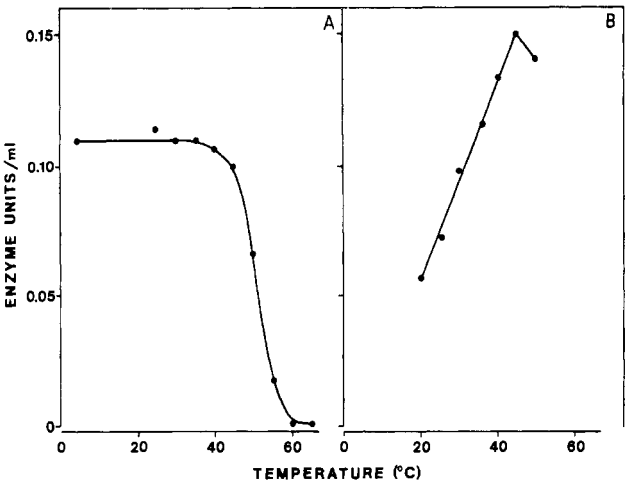

Figure 5. (A) Heat sensitivity of proteolytic activity. Portions of the final enzyme preparation were held at the temperature indicated for 20 min at pH 8, rapidly chilled, and then assayed at 37 °C for activity. (B) Determination of temperature optima. All assay mixtures were equilibrated at the temperature prior to the addition of the substrate. Activity is expressed as enzyme units. The enzyme units are defined in Table I.

Table II. Effect of Various Compounds on the Activity of Alfalfa Neutral Protease

compound	final concn, mM	rel act., %
none		100
Cu^{2+}	0.1	98
Ni^{2+}	0.1	97
Pb^{2+}	0.1	87
Zn^{2+}	0.1	114
Cs^{+}	0.1	106
Mn^{2+}	0.1	106
Li^{+}	0.1	100
Mg^{2+}	0.1	106
Fe^{2+}	0.1	106
Fe^{3+}	1	92
Cd^{2+}	0.1	104
Co^{2+}	2	88
Ca^{2+}	0.1	104
PCMB	0.1	100
β-mercaptoethanol	1	100
EDTA	3	97
PMSF	0.35	42
Leupeptin	5×10^{-8}	50
reduced glutathione	2	88

concentration of serine protease inhibitors such as PMSF (Fahrney and Gold, 1963) and Leupeptin (Ikezawa et al., 1972; Umezawa, 1972; Kuramoki et al., 1979) as shown in Table II.

The inhibition curve of proteolytic activity by Leupeptin, reported in Figure 6, shows a hyperbolic shape with 50% inhibition at a Leupeptin concentration of 5×10^{-8} M, with an active site concentration of 1.1×10^{-8} M, measured by the method of Chase and Shaw (1970) using *p*-NPGB as the active-site serine titrator.

Two mechanisms by which Leupeptin inhibits proteolytic activity have been proposed which include the formation of hemiacetal or thiohemiacetal between the aldehyde group of Leupeptin and serine hydroxyl or cysteine sulfhydryl groups, respectively (Thompson, 1973; Westerik and Wolfenden, 1972; Rawn and Lienhard, 1974; Bendal et al., 1977; Lewis and Wolfenden, 1977; Lowe and Nurse, 1977; Kennedy and Schultz, 1979). In our case the thiohemiacetal formation seems to be excluded on the basis of noninvolvement of cysteinyl residues in the catalysis as shown by the insensitivity to sulfhydryl reagents. On the contrary, the involvement of serine hydroxyl groups in the

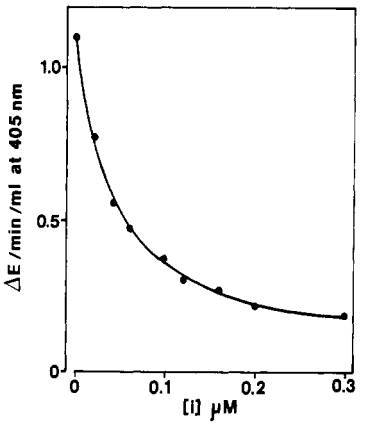

Figure 6. Effect of increasing Leupeptin concentration on proteolytic activity. Leupeptin was added simultaneously with the enzyme; the mixtures were incubated at 37 °C for 5 min, and the reaction was started by addition of the substrate. The velocity is expressed as micromoles of substrate transformed per minute per milliliter at 37 °C.

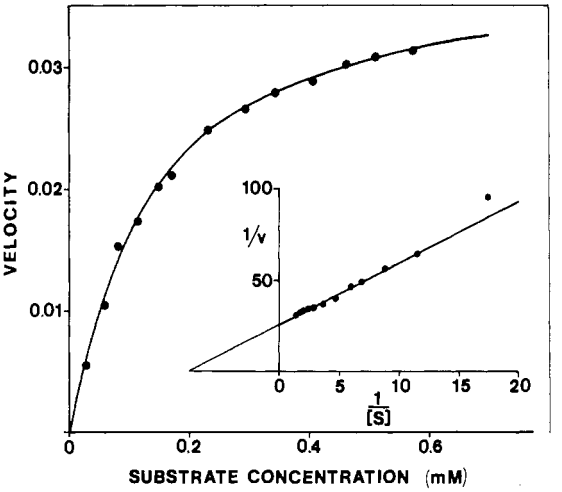

Figure 7. Initial velocity plot of alfalfa neutral protease by using DL-BAPNA as the substrate. The inset shows the plot $1/v$ vs. $1/[S]$. The velocity is expressed as micromoles of substrate transformed per minute per milliliter at 37 °C. K_m = 0.133 mM.

Table III. Effect of Dialysis on Protease Inhibition by Acetylleucylleucylargininal (Leupeptin)

	act., %	
	before dialysis	after dialysis
in the absence of Leupeptin	100	60
in the presence of Leupeptin (1.4×10^{-7} M)	27	58

catalysis, as shown by the Chase and Shaw (1970) titration, suggests the formation of hemiacetal. Further evidence of hemiacetal formation is supported by the fact that inhibition of Leupeptin is removed after dialysis (Table III).

In Figure 7 the V/S and the double-reciprocal plots for DL-BAPNA are reported. The apparent K_m value is 0.13 $\times 10^{-3}$ M, which sensibly approaches the K_m value (0.43 $\times 10^{-3}$ M) of trypsin for the same synthetic substrate (Trautschold et al., 1966).

The insensitivity to thiol reagents, the reversibility of the Leupeptin inhibition, the sensitivity to PMSF and *p*-NPGB, and the K_m value for DL-BAPNA suggest that the proteolytic activity of alfalfa juice is a serine–trypsin-like protease.

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Investigation of Sulfur-Containing Components in Roasted Coffee

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By means of distillation–extraction, adsorption chromatography, and capillary gas chromatography–mass spectrometry, 23 sulfur components (mercaptans, sulfides, and di- and trisulfides) were characterized in roasted coffee. Fifteen components were identified for the first time and confirmed by synthesis (among them are sulfur-containing furans and two dithiolanes). The amounts of sulfur components were determined in roasted Arabica and Robusta coffees, as well as in instant coffee.

More than 100 sulfur-containing components (among them are mercaptans, sulfides, disulfides, thiophenes, and thiazoles) were identified in roasted coffee. Reichstein and Staudinger (1926) characterized furfurylmercaptan (2-furylmethanethiol) as an important aroma constituent of roasted coffee which is considered to be a character impact component (Ohloff and Flament, 1978). Kahweofuran (2-methyl-3-oxa-8-thiabicyclo[3.3.0]-1,4-octadiene) was isolated from coffee by Stoll et al. (1967) and its structure confirmed by synthesis (Büchi et al., 1971). According to Ohloff and Flament (1978), kahweofuran possesses a violent sulfury odor in the pure state and develops a pleasant roasted and smoky note in high dilution. Dimethyl sulfide, methyl ethyl sulfide, furfuryl methyl sulfide, 5-methylfurfuryl methyl sulfide, difurfuryl sulfide, and 15 thiophenes were characterized by Stoll et al. (1967). Vitzthum and Werkhoff (1974) detected 25 thiazoles in aroma concentrates from roasted coffee. In the present work, we report the identification and (semi)quantification of 23 mercaptans, sulfides, and di- and trisulfides in roasted Arabica and Robusta coffee. Fifteen components were characterized for the first time and their structures confirmed by synthesis.

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EXPERIMENTAL SECTION

Materials. High-grown Arabica coffee of Columbian origin and Robusta coffees were roasted to a medium roast and stored in packages with an excess of air at 4 or 25 °C.

Isolation of Sulfur Components. One hundred grams of roasted coffee was ground and placed in a 2-L round-bottom flask containing 1 L of distilled water. The pH was 4.7–5.0. The volatiles were isolated by distillation–extraction (Schultz et al., 1977) with 100 mL of freshly distilled ether–pentane (1:1) for 2 h. The extract was dried over anhydrous Na_2SO_4, concentrated to a volume of 0.1 mL by using a special holdup Vigreux column, stored under nitrogen at −10 °C, and further separated by ad-

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