Isolation and Partial Characterization of Grape Aminopeptidase

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Aminopeptidase from grape berries was purified to homogeneity and partially characterized. The enzyme preparation was stable for several months in 10% glycerol. Aminoacyl-β-naphthylamides as well as aminoacyl-*p*-nitroanilides and dipeptides could be hydrolyzed by the enzyme but Leu-Gly-Gly, Gly-Gly-Leu, Leu-Gly-Gly-Gly, and carboxypeptidase substrates were not cleaved. The enzyme gave very limited hydrolytic products from casein and grape protein. Its temperature and pH optima were 40 °C and 7.4, respectively, and the activation energy was 9.5 kcal/mol. SH agents, S–S reducing agents, and diphenylcarbamyl chloride inhibited to varying extents its activity, whereas phenylmethanesulfonyl fluoride exhibited a poor effect. The molecular weight of the enzyme was estimated to be 95 000. By disc electrophoresis, $NaDodSO_4$ electrophoresis, and isoelectric focusing on polyacrylamide gels, two variants (M_r 62 000 and 33 000) were detected. These properties of the enzyme were compared with those of other plant aminopeptidases.

The proteolytic enzymes of grape berries are thought to be involved in the hydrolysis of grape proteins during fermentation of juice (Neubeck, 1975) as well as during anaerobic fermentation (Cordonnier and Dugal, 1968; Ribéreau-Gayon et al., 1976) and thermovinification of grape (Cordonnier and Dugal, 1968). These involvements, however, remain to be established.

Our studies have focused on the properties of these enzymes. In a preliminary communication (Pallavicini and Dal Belin Peruffo, 1977), two of us reported the presence of three proteolytic activities in grape berries. This paper describes some properties of a homogeneous preparation of grape aminopeptidase.

EXPERIMENTAL SECTION

Plant Material. Mature grape clusters, *Vitis vinifera* var. Riesling, from a local vineyard, were washed, packed, and stored according to the procedure of Arnold (1965).

Enzyme Purification. *Step 1: Crude Extract.* A 250-g sample of frozen berries (minus seeds) was homogenized for 3 min at full speed in an Ultra Turrax homogenizer with 0.5 mL/g of berries of the following prechilled solution, pH 8.8: 0.2M Tris–glycine buffer, polyclar AT (0.1 g dry equiv/g of berries) previously swelled, and 0.5% ascorbic acid. To the slurry was added 0.2% (v/v) of Triton X-100, and then the mixture was stirred overnight. The stirred homogenate was strained through four layers of cheesecloth and the filtrate recovered.

Step 2: Ammonium Sulfate Treatment. The proteins were precipitated from the above filtrate with $(NH_4)_2SO_4$ to 70% saturation, collected by centrifugation at 37000*g* for 20 min, and resuspended in ~420 mL of the above solution, pH 8.8, containing 0.1% Triton X-100. The resulting suspension was gently shaken overnight and then filtered through cheesecloth as before, and the resulting filtrate recovered.

Step 3: DEAE-Sephadex (Batch). The above filtrate was dialyzed against 6 L of 0.02 M Tris-HCl buffer, pH 7.4, centrifuged at 37000*g* for 20 min, and then added to 2 g/100 mL DEAE-Sephadex A-50 previously equilibrated in the above buffer. The slurry was stirred for 1 h at room temperature, filtered, and washed with the buffer solution. After exhaustive washing, the exchanger was further washed with 200 mL of the same buffer containing 0.25 M NaCl. The aminopeptidase was desorbed with 200 mL of the above buffer containing 0.5 M NaCl.

Step 4: Sephadex G-200 Fractionation. The eluate from step 3 was concentrated to 20 mL against poly(ethylene glycol), M_r ~40 000, dialyzed against 0.05 M Tris-HCl buffer, pH 7.4, containing 10% glycerol, and applied on a Sephadex G-200 column (2.6 × 90 cm) equilibrated with 0.05 M Tris-HCl, pH 7.4. Proteins were eluted with the same buffer, and 10-mL fractions were collected.

Step 5: Sephadex G-100 Fractionation. Active fractions from step 4 were pooled, concentrated as before, and applied on a Sephadex G-100 column (1.6 × 60 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 7.4. Proteins were eluted with the same buffer, and 5-mL fractions were collected.

Step 6: DEAE-Sephadex A-50 Column. Active fractions from step 5 were pooled, dialyzed against 0.02 M Tris-HCl buffer, pH 7.0, and applied on a DEAE-Sephadex A-50 column (1.6 × 30 cm) equilibrated with the same buffer. After exhaustive washing of the column, adsorbed aminopeptidase was eluted with 300 mL of a linear gradient of NaCl up to 0.5 M, and 3.7-mL fractions were collected. The pooled enzyme fractions (28 mL) were dialyzed against 0.05 M Tris-HCl buffer, pH 7.4, and used either for estimation of purity of the enzyme or for characterization experiments.

Protein Determination. Protein in enzyme preparations was measured by a modified Lowry procedure (Madsen, 1969) with bovine serum albumin as the standard. Protein concentration in column effluents was estimated from the absorbance at 280 nm.

Enzyme Assays. All activity assays were carried out at pH 7.4, which represents the optimum pH for the pure enzyme (data not shown), in accordance with the results obtained with a partially purified enzyme preparation (Pallavicini and Dal Belin Peruffo, 1977).

Aminopeptidase activity was routinely measured by using 0.1 mL of the enzyme solution, 0.8 mL of 0.05 M Tris-HCl buffer, pH 7.4, and 0.1 mL of 1 mM L-alanine-β-naphthylamide (Ala-β-Na) in methanol. After incubation at 38 °C for 50 min, the reaction was stopped and the free β-naphthylamide was kept in solution by the addition of 3 mL of absolute ethanol (Exterkate, 1973). A standard curve was prepared by using the β-naphthylamide solution. Under this assay condition, enzyme activity was a linear function of both incubation time and enzyme concentration (Figure 1). The Ala-β-Na substrate was also used to localize enzyme-containing fractions in column effluents.

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Table I. Purification of Aminopeptidase from Grape Berries

steps	vol, mL	act., units/mL	total units	protein, mg/mL	sp act., units/mg	purification	yield, %
initial extract	1.350	0.34	459	0.73	0.46		100
$(NH_4)_2SO_4$ ppt (0.70 sat.)	450	0.89	400	1.61	0.55	1.19	87.1
DEAE-Sephadex (batch)	200	1.48	296	0.61	2.43	5.28	64.5
Sephadex G-200	130	2.10	273	0.53	3.96	8.61	59.5
Sephadex G-100	48	4.77	229	0.33	14.45	31.41	49.9
DEAE-Sephadex (column)	26	5.71	148	0.08	71.37	155.15	32.2

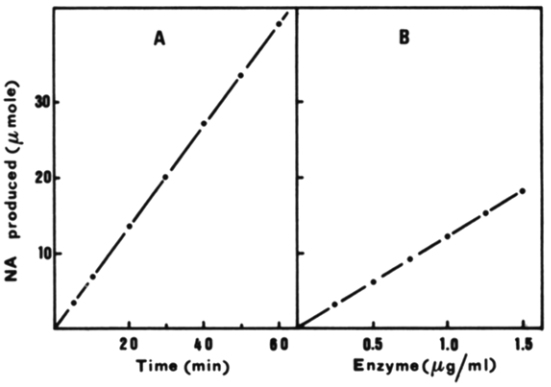

Figure 1. (A) Aminopeptidase activity as a function of time; (B) effect of aminopeptidase concentration on the initial rate of hydrolysis of ANA.

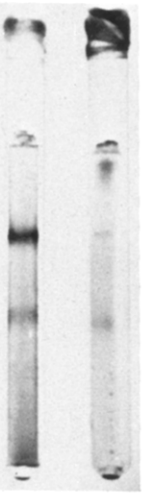

Figure 2. Disc gel electrophoresis of the purified grape aminopeptidase. A sample of the purified enzyme (50 μg) was applied to a gel column of 12% polyacrylamide and run at pH 8.3 for ~4 h at 2 mA/ column. Enzyme activity was detected by incubation of the gel in 0.2 M Tris–maleate buffer, pH 6.0, containing 50 mg of Black K salt and 40 mg of Ala-β-Na (Beckman et al., 1964). Gels were stored in a mixture of methanol, acetic acid, and water in the ratio 5:5:1. Protein was stained by Amido Black for 1 h and destained overnight in 7.5% acetic acid. The direction of electrophoresis was from top to bottom. Left column: aminopeptidase activity staining. Right column: protein staining.

Detection of aminopeptidase activity on gels from disc electrophoresis was achieved with the method of Beckman et al. (1964). Activity on L-alanine-*p*-nitroanilide (ANA) or L-leucine-*p*-nitroanilide (LNA) was followed by using 1 mM substrate in 0.05 M Tris-HCl buffer, pH 7.4. The increase in absorbance was determined at 410 nm, and the change in absorbance was converted to concentration by a molar extinction coefficient of 10 000 M^{-1} cm^{-1} for NA (Pfleiderer, 1970). A unit of activity was defined as that amount of enzyme which liberated 1 μmol of product from the substrates min^{-1} (mL of enzyme preparation)$^{-1}$. The specific activity was calculated by relating the enzyme units to 1 mg of protein. The hydrolysis of peptides was followed by estimation of the liberated amino acids with 2,4,6-trinitrobenzenesulfonic acid (Kolehmainen and Mikola, 1971). Carboxypeptidase activity was determined on carbobenzoxyleucyltyrosine (Z-Leu-Tyr) and carbobenzoxyphenylalanylalanine (Z-Phe-Ala) as the substrate (Tschesche and Kupfer, 1972). Esterase activity was tested with *N*-benzoyl-L-arginine ethyl ester (BAEE) (Arnon, 1970), while amidase activity was determined on benzoyl-DL-arginine-*p*-nitroanilide (BAPA) (Arnon, 1970). Endopeptidase activity was determined on casein (Hammarsten grade) and on native grape protein. The reaction mixtures were incubated at 38 °C for 60 min, and then the nonhydrolyzed protein was precipitated with 10% Cl_3Ac-OH and filtered off, and the absorbance increase of the filtrate measured at 280 nm (Ohmiya et al., 1978).

Grape Protein Preparation. From 200 mL of fresh prepared grape juice, proteins were precipitated with ammonium sulfate (70% saturation). Precipitated proteins were collected by centrifugation at 37000*g* for 20 min, redissolved in water, and then dialyzed against several changes of distilled water. The content of the dialysis bag was centrifuged at 2000*g* for 10 min to remove insoluble material, and the supernatant freeze-dried. The freeze-dried material was dissolved in 0.05 M Tris-HCl buffer, pH 7.4, and used at 0.5% ($N \times 6.25$) concentration of protein solids as the substrate for enzyme activity.

Electrophoresis. For estimation of purity of the enzyme preparations, disc gel electrophoresis was performed in 7.5% polyacrylamide gels using Tris–glycine buffer, pH 8.3 (Davis, 1964). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed to determine the molecular weight of subunits of the purified enzyme under essentially the same conditions as those of Weber and Osborn (1969). Isoelectric focusing in 7.5% polyacrylamide gels was carried out as previously reported (Pallavicini and Dal Belin Peruffo, 1975; Dal Belin Peruffo and Pallavicini, 1975).

RESULTS

Enzyme Purification. Table I shows the purification of aminopeptidase. The 155-fold purified enzyme was free from contaminating protein as ascertained by disc gel electrophoresis, and the final preparation showed two bands of activity (Figure 2).

Enzyme Properties. *Stability.* When stored in the absence of protective reagents the homogeneous enzyme was rather unstable. Little or no activity was observed after 6–8 days at 4 °C. In the presence of 10% glycerol, solutions of aminopeptidase in 0.05 M Tris-HCl buffer, pH 7.4, could be kept at 4 °C for several months without loss of activity.

Specificity. The purified enzyme was poorly active on casein and on grape protein and had no amidase or carboxypeptidase activity (Table II). The enzyme hydrolyzes dipeptides but no tripeptides and Leu-Gly-Gly-Gly.

The hydrolysis of dipeptides was studied only for substrates that were anticipated to be reactive on the basis of naphthylamide substrate specificity (Pallavicini and Dal Belin Peruffo, 1977). The relative aminopeptidase activities among various dipeptides were unlike from amino-

Table II. Substrate Specificity of Grape Aminopeptidase

substrate	μM min^{-1} × 10^2
Ala-β-Na	6.0
Leu-β-Na	2.9
Phe-β-Na	0.4
ANA	4.9
LNA	2.8
Ala-Gly[a]	4.1
Ala-Met[a]	4.1
Leu-Tyr[a]	7.4
Gly-Phe[a]	1.0
Glu-Leu[a]	0.5
Gly-Gly-Leu	0
Leu-Gly-Gly	0
Leu-Gly-Gly-Gly	0
Z-Leu-Tyr	0
Z-Phe-Ala	0
BAEE	2.3
BAPA	0
casein (Hammarsten)	0.02
grape protein	0.035

[a] Values are approximate since the method detects both amino acids.

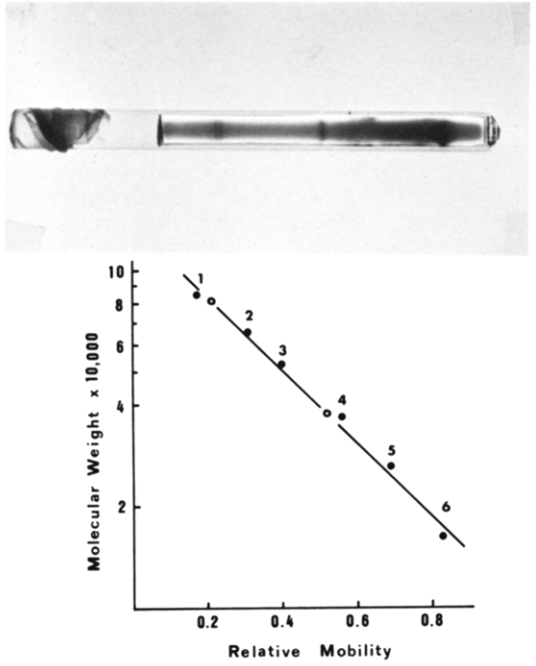

Figure 3. NaDodSO$_4$ gel electrophoresis of the purified grape aminopeptidase. 100 μg of purified enzyme was reduced and applied on 5% gels. The direction of electrophoresis was from left to right. The molecular weights of marker proteins were plotted on a logarithmic scale against their mobilities relative to the tracking dye. (1) Bovine albumin (M_r 66 000); (2) egg albumin (M_r 45 000); (3) pepsin (M_r 34 700); (4) chymotrypsinogen (M_r 24 000); (5) β-lactoglobulin (M_r 18 400); (6) lysozyme (M_r 14 300).

acylnaphthylamides. For example, dipeptides with an N-terminal alanine gave lower rates of hydrolysis than those with an N-terminal leucine.

Isoelectric Point. The apparent isoelectric point of the fast moving and slow moving band (Figures 2 and 3) was 4.4 and 5.2, respectively.

Molecular Weight. When the enzyme was subjected to NaDodSO$_4$–polyacrylamide gel electrophoresis with or without prior reduction with 2-mercaptoethanol showed two bands of protein having molecular weights of 63 000 and 32 000 (Figure 3). The sum of the molecular weights of the two bands gave a total molecular weight of ~95 000. This value was in good agreement with the result of gel filtration on Sephadex G-100 column in which the enzyme

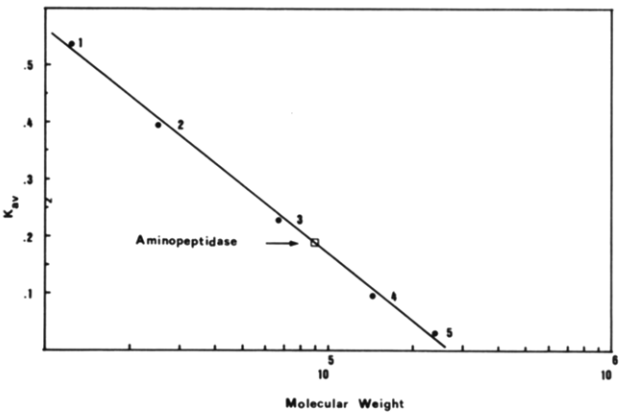

Figure 4. Molecular weight determination of grape aminopeptidase on the Sephadex G-100 column. The column (1.6 × 60 cm) was equilibrated with 0.1 M Tris-HCl buffer, pH 7.4. (1) Cyctochrome *c* (M_r 12 400); (2) chymotrypsingoen A (M_r 25 000); (3) bovine serum albumin (M_r 67 000); (4) aldolase (M_r 147 000); (5) catalase (M_r 240 000). V_0 and V_t of the column were calculated with blue dextran and $K_2Cr_2O_5$, respectively.

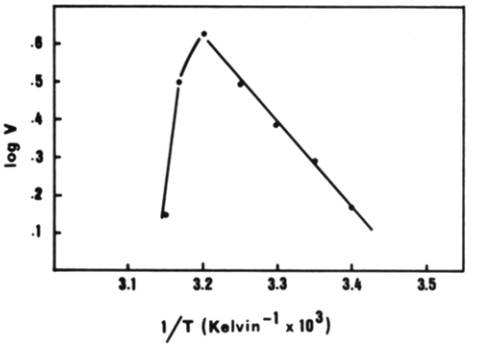

Figure 5. Arrhenius plot of grape aminopeptidase using ANA as the substrate in 0.05 M Tris-HCl buffer, pH 7.4.

behaved as a single component in terms of enzyme activity with a k_{av} of 0.185 when the aminopeptidase peak was plotted on a selectivity curve for the column (Figure 4).

Rate Dependence on Temperature. The effect of temperature on aminopeptidase activity is shown in Figure 5. From the Arrhenius plot it can be noted that the activation energy of the enzyme on Ala-β-Na was 9.5 kcal/mol. The optimum temperature was 40 °C. The Q_{10} value was 1.75 between 30 and 40 °C and 1.65 between 20 and 30 °C.

Heat Inactivation. The kinetics of heat inactivation of grape aminopeptidase at 40, 45, 50, and 55 °C are shown in Figure 6. The logarithmic decrease of activity with increase in heating time indicates that the thermal inactivation of the enzyme followed first-order kinetics and was monophasic for all temperatures tested. At 40 °C for 50 min the enzyme retained only 85% of its original activity. At 55 °C, over 90% of original activity was lost after 25-min preincubation.

Effect of Various Reagents on Enzyme Activity. Preincubation of the enzyme with SH blocking reagents such as *p*-(chloromercuri)benzoate, iodoacetamide, mercuric acetate, and *N*-ethylmaleimide markedly inhibited the aminopeptidase activity (Table III). Thus, this enzyme seems to have functional SH group(s). Preincubation of the enzyme with mercaptoethanol, dithiothreitol or cysteine likewise inhibited the enzyme, thereby showing that intact disulfide groups are essential for enzyme activity. Dialysis of the cysteine-reduced enzyme against 0.05 M Tris-HCl buffer, pH 7.4, for 24 h at 4 °C, fully restored the enzyme activity. EDTA, ethanol, and methanol had no effect on the activity, whereas ascorbic acid and sodium

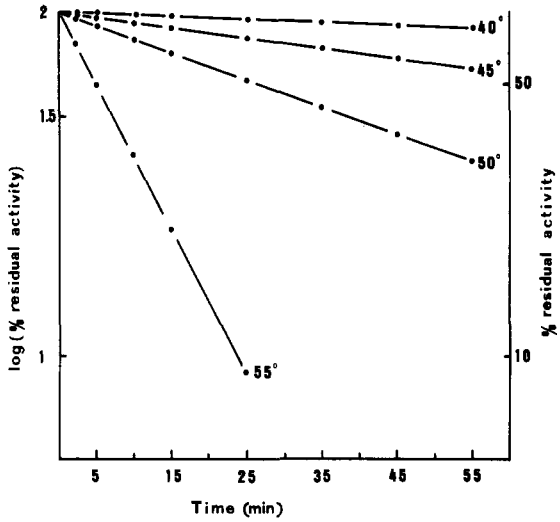

Figure 6. Heat inactivation of grape aminopeptidase. Aliquots of the enzyme solution were preincubated for the prescribed times at the indicated temperatures and rapidly cooled in crushed ice, and the residual activity was measured at 38 °C. The reaction mixtures contained 0.1 mL of enzyme solution, 0.8 mL of 0.05 M Tris-HCl buffer, pH 7.4, and 0.1 mL of 1 mM Ala-β-Na as described in the text.

Table III. Effect of Various Reagents on Enzyme Activity[a]

reagent	concn,[b] mM	rel act.
none		100
p-(chloromercuri)benzoate	0.1	15
p-(chloromercuri)benzoate	1	2
iodoacetate	50	29
iodoacetamide	50	49
mercuric acetate	5	0
N-ethylmaleimide	5	23
dithiothreitol	1	44
dithiothreitol	10	7
2-mercaptoethanol	1	42
2-mercaptoethanol	5	0
cysteine	1	79
cysteine	5	0
phenylmethanesulfonyl fluoride	1	86
diphenylcarbamyl chloride	1	25
EDTA	1	95
EDTA	5	89
$NA_2S_2O_5$	10	0
ascorbic acid	1	0
ethanol	100	93
methanol	100	95

[a] Aliquots of enzyme preparation were pretreated separately with the reagent listed for 10 min prior to the assay. [b] All solutions were freshly prepared and water was the solvent except for phenylmethanesulfonyl fluoride which was dissolved in ethanol. The final ethanol concentration for preincubation was 0.2%.

metabisulfite at 1 mM concentration strongly inhibited the enzyme. Phenylmethanesulfonyl fluoride, an inhibitor of serine proteases, showed a small inhibitory effect, and diphenylcarbamyl chloride, which reacts with histidine residues, was more strongly inhibitory.

Comparison with Other Plant Aminopeptidases. Among the aminoacyl-β-naphthylamides tested, Ala-β-Na is a better substrate than Leu-β-Na or Phe-β-Na, in accord with the results obtained with the partially purified grape berry aminopeptidase (Pallavicini and Dal Belin Peruffo, 1977). The enzyme also acts on dipeptides and aminoacyl-*p*-nitroanilides, in agreement with aminopeptidases of other sources (Doi et al., 1980; Du Toit and Schabort, 1978a). But, in contrast to these enzymes, our enzyme is unable to cleave Leu-Gly-Gly or Gly-Gly-Leu. For its lack of activity on tri- or tetrapeptides or BAPA, it differs also from other known aminopeptidases (Kolehmainen and Mikola, 1971; Schabort and Du Toit, 1978; Sopanen and Mikola, 1975; Ashton, 1976; Catsimpoolas et al., 1971). Its optimum temperature is slightly lower than that of *Cucurbita Maxima* cotyledons (Ashton and Dahmen, 1968), germinating barley (Burger et al., 1970), and *Agave americana* (Du Toit and Schabort, 1978b) aminopeptidases. Moreover, the grape enzyme differs from the *A. americana* one in both activation energy and Q_{10} values. By disc electrophoresis, $NaDodSO_4$ electrophoresis, and isoelectric focusing on polyacrylamide gels, two variants of activity could be detected for the enzyme reported here. Thus, the enzyme exists as a mixture of two forms similar to the germinating seeds of *Pinus sylvestris* (Salmia and Mikola, 1976), wheat kernels (Kruger and Preston, 1978), castor bean endosperm (Tully and Beevers, 1978), and pea (Elleman, 1974) aminopeptidases but differs from that of *A. americana* (Du Toit et al., 1978) which migrates as a single protein band and from that of *Picea abies* needles (Lundkvist, 1974), that of maize endosperm (Beckman et al., 1964), and that of peanuts (Thomas and Neucere, 1973; Cherry et al., 1973) since these are electrophoretically more heterogeneous enzymes. The isoelectric point of both forms of grape aminopeptidase agrees enough well with that of *A. americana* (Du Toit et al., 1978) with a p*I* of 4.53 and that of soybean seeds (Catsimpoolas et al., 1971) with a p*I* of 4.80.

The results of inhibitor study tend to suggest that the grape enzyme is a thiol protease. In this respect it behaves like that of *Zea mays* seedling (Feller et al., 1978), that of castor bean endosperm (Tully and Beevers, 1978), and the AP2 of pea (Elleman, 1974), which are inactivated by both *p*-(chloromercuri)benzoate and *N*-ethylmaleimide but differs from LAPase of pea (Tomomatsu et al., 1978) and aminopeptidases of other sources (Du Toit and Schabort, 1978a; Schabort and Du Toit, 1978; Sopanen and Mikola, 1975). The grape enzyme is not effected by EDTA. This result is dissimilar from that reported for aminopeptidases of rice (Doi et al., 1980) and *C. maxima* cotyledones (Ashton and Dahmen, 1967) but consistent with the results found for other similar enzymes (Elleman, 1974; Ashton and Dahmen, 1968; Scandalios and Espiritu, 1969; Tomomatsu et al., 1978). The molecular weight of our enzyme is larger than that of *A. americana* aminopeptidase (Du Toit et al., 1978) but smaller than that of 132000 (M_r of AP1 plus M_r of AP2) reported for pea aminopeptidase (Elleman, 1974).

DISCUSSION

Our biochemical characterization of grape aminopeptidase was particularly aimed at understanding the role it might play in hydrolyzing grape protein during processing of the grape. Among the substrates tested, the pure enzyme had maximal activity against Ala-β-Na, ANA, and Leu-Tyr. It displayed negligibly low activity on proteins and no carboxypeptidase or esterolytic activity, and at temperatures above 40 °C its activity was significantly diminshed. Furthermore, the optimum pH of the enzyme was much higher than the normal pH of grapes. According to these observations, it seems that grape aminopeptidase may play only a minor role in grape protein hydrolysis during the processing of grape or grape juice to wine. Grape berries contain other proteolytic enzymes such as an endopeptidase showing maximum activity at about pH 2 on hemoglobin (Cordonnier and Dugal, 1968) and a carboxypeptidase with an optimum pH around 4.5 on Z-Leu-Tyr (Pallavicini and Dal Belin Peruffo, 1977) which

may be of major importance in this respect.

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Partial Purification and Properties of a Hydroperoxide Lyase from Fruits of Pear

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A relatively stable hydroperoxide lyase was partially purified from pears by differential centrifugation, gel chromatography, and isoelectric focusing. The enzyme preparation was optimally active at pH 6.5. It was specific for 9-hydroperoxide isomers of linoleic and linolenic acid. The former substrate was cleaved to *cis*-3-nonenal and 9-oxononanoic acid.

The formation of volatile aldehydes of chain lengths C_6 and C_9 by an enzymatic oxidative cleavage of a C_{18} unsaturated fatty acid containing a *cis*-1,*cis*-4-pentadiene system is widespread in fruits and vegetables (Gardner, 1975; Hatanaka et al., 1978; Tressl and Drawert, 1973). The process proceeds rapidly when plant cells are disrupted in the presence of oxygen. Linoleic and linolenic acids, liberated from endogenous lipids by acyl hydrolases, are converted to their hydroperoxides by lipoxygenase enzyme (Wardale and Galliard, 1975). In watermelon seedlings (Vick and Zimmerman, 1976), tomato fruits (Galliard and Matthew, 1977), tea chloroplasts (Hatanaka et al., 1979), bean leaves (Matthew and Galliard, 1978), and alfalfa seeds (Sekiya et al., 1979), a hydroperoxide lyase has been detected which subsequently cleaves the 13-hydroperoxides in C_6 aldehydes and 12-oxo-*cis*-9-dodecenoic acid. Hexanal is formed from 13-hydroperoxyoctadeca-*cis*-9,*trans*-11-dienoic acid (13-HPOD) and *cis*-3-hexenal from 13-hydroperoxyoctadeca-*cis*-9, *trans*-11,-*cis*-15-trienoic acid (13-HPOT).

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A hydroperoxide lyase reacting with both 9- and 13-hydroperoxides occurs in fruits of cucumbers (Galliard et al., 1976). As well as 9-oxononanoic acid, *cis*-3-nonenal is released from 9-hydroperoxyoctadeca-*trans*-10,*cis*-12-dienoic acid (9-HPOD) and *cis*-3,*cis*-6-nonadienal from 9-hydroperoxyoctadeca-*trans*-10,*cis*-12,*cis*-15-trienoic acid (9-HPOT). In plant tissues the cis-3 double bond in the enals is often enzymatically isomerized to the conjugated trans-2 derivatives (Phillips et al., 1979).

Hydroperoxide lyases from fruits (Phillips and Galliard, 1978) and seedlings of cucumbers and alfalfa (Vick and Zimmerman, 1976) were partially purified and separated from lipoxygenase. The membrane-bound and extremely heat labile enzyme from cucumber fruits catalyzes the cleavage of hydroperoxides to carbonyl compounds without formation of free intermediates (Phillips and Galliard, 1978).

EXPERIMENTAL SECTION

Materials. Pear fruits (*Pyrus communis* L.) were purchased from local markets; their origin varied seasonably and the cultivars were unidentified. 13-HPOD was prepared from linoleic acid and 13-HPOT from linolenic acid by using purified type I soybean lipoxygenase (Eg-

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