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AN AMINOPEPTIDASE FROM *AGAVE AMERICANA*, ISOLATION AND PHYSICAL CHARACTERIZATION*

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Key Word Index—*Agave americana*; Amaryllidaceae; proteolytic enzyme; aminopeptidase; isolation; purification; properties.

Abstract—A new aminopeptidase was isolated from *Agave americana* by fractionation, chromatography and gel filtration. The mw of the enzyme, determined by different procedures was 86000 ± 1500; the enzyme had a sedimentation coefficient of 4.96 S, a diffusion coefficient of 5.2×10^{-7} cm^2/sec, a Stokes radius of 3.8 nm, a partial specific volume of 0.733 cm^3/g, a frictional ratio of 1.40, a molecular absorbancy index at 280 nm of 8.36×10^4, an isoelectric point of 4.53 and contained 1.25% carbohydrate. The amino acid composition of the enzyme was determined and the aminoterminal residue was identified as lysine whilst the carboxylterminal residue was either leucine or isoleucine. No subunit structure was observed for the enzyme.

INTRODUCTION

It was found that the extract of the leaves of the plant *Agave americana* L. Am. trop. contains proteolytic enzymes. One of these enzymes, an aminopeptidase, was isolated and characterized. This seems to be the first such enzyme from a plant source to be investigated. Aminopeptides have already been obtained from other sources, including higher animals [1–7] and microorganisms [8–12]. Some of these enzymes have relatively high MWs of the same order of magnitude as that of leucine aminopeptidase, whilst a few have MWs about a third of that. All aminopeptidases are also not to the same extent dependent upon participation of a metal ion during catalysis. *Agave* aminopeptidase has a relatively low MW and does not seem to need metal ions for optimal activity. The enzyme was isolated and characterized physically as described in this paper.

RESULTS

Purification of enzyme

The enzyme was isolated and purified according to the procedure as outlined in the Experimental. The results of this procedure are summarized in Table 1, and a unit of enzyme activity is defined as the amount of enzyme that converts 1 μmol substrate to products in 1 min. As an example of the final purification and also the purity of the enzyme, the results as indicated in Figs 1 and 2 are given. These are the flow diagrams for the chromatography steps, steps 5 and 6. By using this procedure 7.4 mg aminopeptidase, purified 921-fold, was obtained from 3.8 kg plant material. The isolation procedure was repeated several times and results were consistent during these different procedures. Dialysis against deionized water for long periods tended to inactivate the enzyme to some extent with the result that where possible, this was avoided. The enzyme was stored in powder form in the cold where necessary, without any significant loss of activity.

Properties of enzyme

Electrophoretic homogeneity. Under the conditions of electrophoresis used, *Agave* aminopeptidase migrated as a single protein band as observed by dyeing with amido black or an affinity dyeing procedure [13, 14]. The enzyme migrated about 25 mm under these conditions. Preparative gel electrophoresis (procedures and results not given) also indicated a homogeneous preparation. Electrophoresis was also carried out under a variety of denaturing conditions, 7 M urea, 3 M guanidinium hydrochloride and 1% sodium lauryl sulphate, and in all cases only single protein bands were observed. These results were in accordance with other results in indicating no subunit structure for the enzyme. Electrophoresis in different buffers at different pH-values also indicated a homogeneous fraction.

Isoelectric point. The isoelectric point of *Agave* aminopeptidase was 4.53 as determined in ampholyte gradients of pH 3 to 10 and pH 4 to 6. The isoelectric point of the enzyme thus falls within the same region as the values for leucine aminopeptidase [15] and *Bacillus subtilis* aminopeptidase [12].

Sedimentation coefficient. Sedimentation studies were conducted with *Agave* aminopeptides at concentrations

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Table 1. Isolation of an aminopeptidase from *Agave americana*. Summary of results of the different purification steps

Step	Total protein (mg)	Total enzyme (units × 10^3)	Specific activity (units/mg protein × 10)	Purification (fold)	Yield (%)
1. Crude extract	21900	14600	0.665	1	100
2. 40–60% $(NH_4)_2SO_4$ precipitate	3280	11900	3 62	5.45	82
3. DEAE–Sephadex A-25 eluate	20.4	6530	320	481	45
4. Sephadex G-200 eluate	9.5	5660	600	902	39
5. CM–Sephadex C-25 eluate	8.0	4900	612	921	34
6. Sephadex G-200 eluate	7.4	4540	612	921	31

ranging from 0.5 to 0.7 mg/ml with buffer concentrations ranging from 0.01 to 0.17 M. A corrected $S_{20,w}$-value of 4.96 S was obtained and no concentration dependence was observed. Results of zone centrifugation studies on sucrose gradients indicated a sedimentation coefficient of 4.8–5.2 S which agreed well with the above-mentioned value.

Diffusion coefficient. The corrected diffusion coefficient of *Agave* aminopeptidase was 5.2 ± 0.15 × 10^{-7} cm^2/sec as determined with the ultracentrifuge. The diffusion coefficient as determined by gel filtration [16] was 5.5 × 10^{-7} cm^2/sec. The standards used were as indicated in the Experimental.

Other physical constants. By using the data as obtained during gel filtration studies, other physical constants were also determined [16]. The enzyme had a Stokes radius (molecular size) of 3.8 nm and a frictional ratio, f/f_0 of 1.40. This last value indicated that *Agave* aminopeptidase is a globular protein. The partial specific volume of the enzyme, as determined from the amino acid composition [17], was 0.733 cm^3/g.

MW. By using the data concerning sedimentation coefficient, diffusion coefficient and partial specific volume, the MW of the enzyme was calculated to be 86 5000 ± 1500 [18, 19]. With gel filtration studies, a value of 87 500 was obtained [16].

Amino acid composition. The amino acid composition of *Agave* aminopeptidase is indicated in Table 2. The enzyme contained a very small amount of cysteine and corrections were made for any losses of specific amino acids. The MW of the enzyme calculated from amino acid composition was 87510. The tryophan content of the enzyme was determined by the procedure of Bencze and Schmid [20] and also by a procedure based on the molar absorbancy indexes of tyrosine, trytophan and other known proteins [21].

Aminoterminal and carboxylterminal groups. According to the procedures used, the aminoterminal amino acid was identified as lysine, whilst the carboxylterminal group was either leucine or isoleucine. Endgroup determinations indicated no subunit structure for the enzyme.

Other chemical properties. The carbohydrate content of *Agave* aminopeptidase, determined by the anthrone method, was 1,25% on wt basis. The carbohydrate fraction was not characterized further. Determination of free sulfhydryl groups with Ellman's reagent under denaturing conditions indicated a value 0.5 moles free sulfhydryl per mole protein. This value agree well with the amino acid analysis values for cysteine.

Absorbancy properties. The enzyme had a molar absorbancy index of 8.36 × 10^4 at 280 nm and a $E^{1\%}_{1\,cm}$-value of

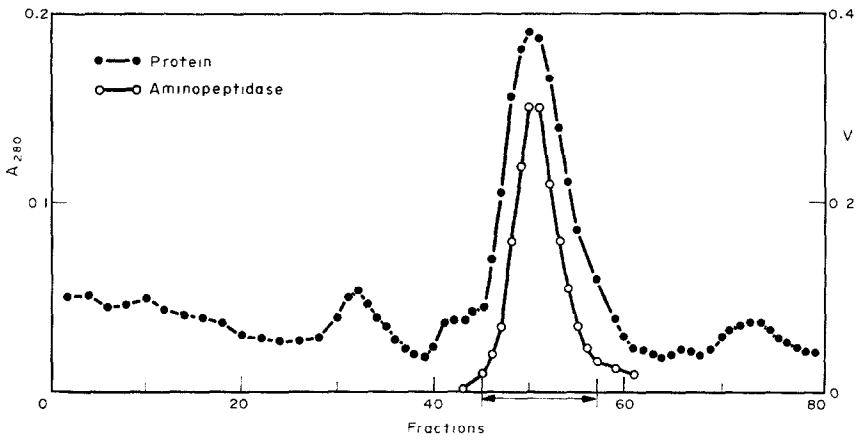

Fig. 1. Chromatography of *Agave* aminopeptidase on a 2.5 × 38 cm CM–Sephadex column. Sample from step 4. Conditions as indicated in text. Fractions were combined as indicated (|↔|) and used for further purification
v = aminopeptidase activity, units/ml, A_{280} = absorbancy at 280 nm.

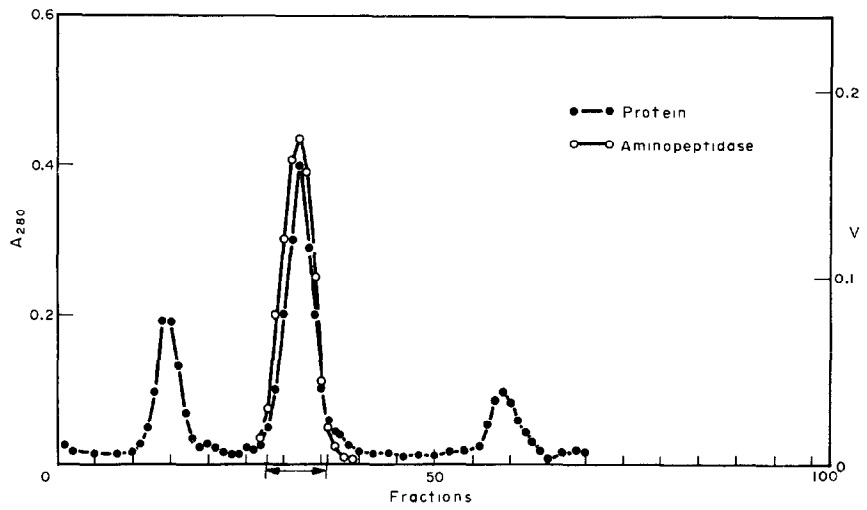

Fig. 2. Chromatography of *Agave* aminopeptidase on a 2.5 × 93 cm Sephadex G-200 column. Sample from step 5, purified enzyme. Conditions similar to those described by Andrews [20]. Fractions were combined as indicated (|↔|) and used for characterization studies. v = aminopeptidase activity, units/ml, A_{280} = absorbancy at 280 nm.

9.7 at 280 nm. The UV spectrum displayed a maximum value at 278 nm and a slight shoulder at 292 nm. The physical properties of *Agave* aminopeptidase are summarized in Table 3.

DISCUSSION

The aminopeptidase isolated from *Agave americana* is the first well-characterized aminopeptidase obtained from a plant. Isolation of the enzyme was possible using classical procedures, but due to the relatively low yield of the enzyme, the isolation procedure was repeated several times to obtain enough enzyme for characterization. The presence of other proteolytic enzymes also caused some problems especially at earlier stages of the procedure. Prolonged dialysis against deionized water partly inactivated the enzyme. The enzyme was obtained in homogeneous form according to purification to a constant specific activity, as well as other homogeneity determinations, which were all repeated several times under varying conditions. The enzyme was thus homogeneous with respect to gel electrophoresis, isoelectric focusing, gel filtration and ultracentrifugal analysis. All physical constants were determined by repeated experiments and the average values of these determinations are indicated in Tables 2 and 3. *Agave* aminopeptidase is amongst the smaller of the known aminopeptidases, with a MW of 86 500. In this respect the enzyme is very similar to the aminopeptidases observed in mammalian brain and *Bacillus subtilis* [12]. These enzymes are very much smaller than leucine aminopeptidase (MW = 3.26×10^5) [22] and other similar aminopeptidases.

Endgroup determinations as well as electrophoretic studies under denaturing conditions indicated no subunit structure for the enzyme. The procedure of affinity-dyeing was successful in not only proving homogeneity with respect to catalytic properties, but also in observing

Table 2. Amino acid composition of *Agave* aminopeptidase

Amino acid	Calculated number of residues/mol protein	Number of residues/mol protein, values rounded off	Number of residues/100 residues
Glutamic acid	73.08	73	10.47
Lysine	47.80	48	6.89
Histidine	6.40	6	0.86
Arginine	25.44	25	3.59
Aspartic acid	72.72	73	10.47
Threonine	51.80	52	7.46
Serine	67.44	67	9.61
Proline	8.16	8	1.15
Glycine	82.56	83	11.91
Alanine	49.44	49	7.03
Cysteine	1.48	2	0.29
Valine	42.24	42	6.03
Methionine	4.00	4	0.57
Isoleucine	34.24	34	4.88
Leucine	77.36	77	11.05
Tyrosine	13.76	14	2.01
Phenylalanine	28.24	28	4.02
Trytophan*	11.80	12	1.72
Total	697.96	697	100.01

*Trytophan was determined spectrophotometrically [20,21]

Table 3. Physical constants of *Agave* aminopeptidase

Physical constant	*Agave* aminopeptidase
$S_{20,w}$ (sec^{-13})	4.96
$D_{20,w}$ (cm^2/sec)	5.2×10^{-7}
MW (± 3%)	86.5×10^3
Stokes radius (nm)	3.8
f/f_0	1.4
$\bar{v}$ (cm^3/g)	0.733
$E^{1\%}_{1\,cm}$ at 280 nm	9.7
Molar absorbancy index at 280 nm	8.36×10^4
Carbohydrate content (%)	1.25
Isoelectric point (pH)	4.53

the enzyme specifically in the presence of other contaminating proteins. This aided in selection of appropriate conditions during isolation procedures. *Agave* aminopeptidase was further relatively stable and could be stored for considerable periods in the dry form in the cold. However, prolonged storage and prolonged dialysis against deionized water did inactivate the enzyme, and with the loss in activity there was usually an increase in low MW substances which did not sediment under conditions of ultracentrifugal analysis, indicating that the enzyme probably attacks and hydrolyses its own amino-terminal residues. The catalytic properties of the enzyme are reported elsewhere [23].

EXPERIMENTAL

Purification procedures were carried out at 5–6° unless otherwise stated. Sephadex ion exchangers and gels (particle size 40–120 μm) were prepared according to the manufacturer's recommendations and equilibrated with the respective buffers. Buffers were prepared according to ref. [24] and columns were eluted by suction with a peristaltic pump. Before use, buffer was pumped through the columns for 8–16 hr. All chemicals and biochemicals used were analytically pure. Protein concns were determined by the Biuret procedure [25], the Lowry procedure [26] or a procedure based on A measurements [27].

Enzyme activity. The enzyme was routinely assayed using 2 mM leucine-*p*-nitroanilide as substrate at 25°. Tris–maleate buffer, 0.18 M, pH 7.2 was used, and the increase in A at 405 nm was followed after addition of the enzyme soln to the reaction mixture [28].

Purification of enzyme. Preparation of crude extract (Step 1). Plant material (3.8 kg) was used and directly after the leaf was removed from the plant, it was treated in such a way that all the fibres were cut off every 1 to 1.5 cm. The green outer sections were removed and discarded. The white material was pulped in a meat grinder and NaCl added up to a final concn of 0.15 M. After standing for 1 hr the suspension was passed through a fine nylon cloth (rubber gloves were worn) to remove fibrous materials. The extract was collected and centrifuged at 10000 *g* for 20 min. The pH of the clear supernatant fluid, the crude extract, (3.5 l) was adjusted to pH 6.5, and a small sample dialysed against 3 × 1 l. deionized H_2O for 16 hr, for determination of enzymic activity and protein concn.

Ammonium sulphate fractionation (Step 2). Crude extract was fractionated with $(NH_4)_2SO_4$ at pH 6.5 at satn levels of 40 and 60% respectively [29]. After the desired satn level was reached, the mixture was left for 1–2 hr before being centrifuged at 12000 *g* for 30 min. The first protein ppt. (0–40%S) as well as the last supernatant fluid (40–60%S) were discarded, as 82% of the original activity was present in the second ppt. (40–60%S). The ppt. was dissolved in 0.05 M NaPi buffer, pH 6.8, and dialysed against 3 × 2 l. of this buffer for 16 hr.

Chromatography on DEAE–Sephadex (Step 3). A DEAE–Sephadex A-25 column (2.5 × 40 cm) was prepared in 0·05 M NaPi buffer, pH 6.8. The sample from step 2 was pumped onto the column at a flow rate of 30 ml/hr and fractions of 7.5 ml each were collected. Starting buffer was pumped through the column for a further 16 hr, or until the eluate of the column had an A at 280 nm of less than 0.1 The column was then developed by a combined concn and pH-gradient over 500 ml. The starting buffer was 0·05 M NaPi, pH 6·8, and the gradient buffer 0.1 M NaPi, pH 5·9 containing 0·5 M NaCl. Positions of eluted proteins were determined by A at 280 nm and the position of the aminopeptidase by activity determinations. The enzyme was eluted at *ca* 0.25 M buffer, these fractions were combined, dialysed against 2 × 2 l. deionized H_2O for 16 hr and freeze-dried.

Gel chromatography on Sephadex G-200 (Step 4). Dry protein from step 3 was dissolved in 0.05 M NaPi, pH 6.8, and dialysed against this buffer (2 l.) for 8 hr. The column (2.5 × 90 cm) was prepared and equilibrated in this buffer and used at 15–20° at a flow rate of 14 ml/hr. The sample was pumped onto the column developed under these conditions, fractions of 6 ml each were collected. Protein and enzyme were localized as previously. The fractions containing the aminopeptidase were combined, dialysed against 2 × 2 l. deionized H_2O for 16 hr and freeze-dried.

Chromatography on CM–Sephadex (Step 5). A CM–Sepahadex C25 column (2.5 × 40 cm) was prepared in 0.015 M Na-citrate-Pi buffer, pH 6. The dry protein from step 4 was dissolved in 0.015 M Na-citrate-Pi buffer, pH 5, dialysed against 2 × 2 l. of this buffer and pumped onto the column at a flow rate of 18 ml/hr. The column was developed at this same flow rate with pH 5 buffer and fractions of 6 ml each were collected. Positions of proteins and aminopeptidase were determined as described previously. The fractions containing the aminopeptidase were combined, dialysed against 2 × 2 l. deionized H_2O for 16 hr and freeze-dried.

Gel chromatography on Sephadex G-200 (Step 6) The procedure as described in ref. [30] was used. Column dimensions were 2.5 × 90 cm, a flow rate of 14 ml/hr was used and fractions of 4.6 ml each were collected. Application of the sample, determination of the position of the enzyme and concentration procedures were as described previously.

Properties of the enzyme. Electrophoretic homogeneity. Purity of protein fractions and the properties of the enzyme were determined by use of a Pleuger Acrylophor apparatus. Electrophoresis was carried out at 160 V, 5 mA/tube for 1 hr at room temp. Various buffers, pH 5 to 9 with varying ionic strengths, and 5, 7.5 and 10% crosslinked polyacrylamide gels were used. Proteins were visualized in the gels after electrophoresis with either amido black staining or an affinity staining procedure [13]. The stained gels were analysed with a densitometer. *Isoelectric focusing.* A 110 ml electrofocusing column (LKB Instruments) with either 1 or 2% (w/v) ampholyte of pH 3 to 10 and pH 4 to 6 were used [31]. The enzyme was introduced into the combined concn and pH gradient as part of the light soln before the gradient was established in the column. Focusing was conducted for 72 hr at 600 V at 5°. The pH of the collected fractions (1 ml) was determined with a radiometer Model 25 pH meter, protein was determined by A at 280 nm and the enzyme localized by activity measurements.

Gel filtration studies. A Sephadex G-200 column (2.5 × 90 cm) prepared according to refs [16, 30] was used. Calibration of the column was carried out with aldolase, yeast alcohol dehydrogenase, BSA fraction V, egg albumin, α-chromotrypsin, trypsin, ribonuclease, cytochrome *c* and blue dextran.

Ultracentrifugation. A Beckman Model *E* analytical ultracentrifuge was used. Sedimentation studies were conducted at 56000 rpm at about 20° for 1 hr and diffusion studies at 6000 rpm at 5° in 12 mm pathlength standard type cells with Al centrepiece and sapphire windows. Boundary formation was observed with the UV optical system at 270 nm [18, 19] Centrifugation on sucrose gradients was carried out in a SW 39 swinging bucket rotor on a Beckman L4 ultracentrifuge according to the procedures of refs [32, 33]. Yeast alcohol dehydrogenase was used as a standard using the procedure of ref. [33].

Amino acid composition. A Beckman Model 118 amino acid analyser was used. Samples of *ca* 1.5 mg (accurate wt) enzyme were hydrolysed with 6N HCl for periods up to 21 hr [34, 35]. The correction factors for losses of cysteine, threonine, serine and tyrosine were obtained as described by ref. [34]. One micromol of each of these amino acids was added to the protein sample, which was hydrolyzed for 4, 8, 16 and 22 hr at 110°. By extrapolation to zero time correction factors were for cysteine 1.56, for serine 1.17 for threonine 1.07 and for tyrosne 1.16. The determined concn of these amino acids were then multiplied by these factors. Trytophan was determined according to the absorbancy properties of the enzyme as well as those of tyrosine and trytophan [20, 21].

Aminoterminal and carboxylterminal endgroups. The procedures as described in refs [36–38] were used respectively.

Other determinations. The sulfhydryl content of the enzyme was determined with 5,5'-dithiobis-(2-nitrobenzoic acid) under denaturing conditions [39] and the carbohydrate content of the enzyme with the anthrone method [40].

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