Biochimica et Biophysica Acta, 429 (1976) 895—911

BBA 67789

ISOLATION AND PARTIAL CHARACTERIZATION OF A PROTEASE FROM *AGAVE AMERICANA VARIEGATA* *

P.J. DU TOIT **

Department Biochemie, Randse Afrikaanse Universiteit, Johannesburg (Republic of South Africa)

(Received July 28th, 1975)

Summary

A new protease was isolated from an extract of leaves of *Agave americana variegata*. The protease (EC 3.4.-) was purified 565-fold with a yield of 39.5%. The 43.8 mg enzyme had a specific activity of 0.44 units/mg. According to electrophoretic, ultracentrifugal and other physical characterizations the enzyme was homogeneous. The enzyme had a M_R of 57 000, a $S_{20,w}$-value of 4.37 S, a $D_{20,w}$-value of $6.8\text{-}7.0 \cdot 10^{-7}$ cm^2sec^{-1}, a Stokes radius of 3.18 nm, a partial specific volume of 0.735 cm^3g^{-1}, a frictional ratio of 1.25, a molecular absorbancy index at 280 nm of $5.773 \cdot 10^4$, an isoelectric point of 5.25 and contained 8—10% carbohydrate. The enzyme contained no cysteine. Agave protease could hydrolyze a variety of protein substrates although it did have a restricted specificity. It is not a sulphhydryl protease but seems to be an alkaline "serine" protease with an optimum pH of 7.8—8.0. Agave protease had marked esterolytic activity and with Cbz-Tyr-ONp had an apparent Michaelis constant of $0.0345 \cdot 10^{-3}$ M and a V of 1.24 mol substrate/mol enzyme per sec. The enzyme did not need metal ions for optimal activity, monovalent cations did not influence its kinetic parameters, but it was inhibited by cobalt, pClHgBzO$^-$ and TosPheCH_2Cl. With respect to its primary specificity, as well as its pH-dependence there was a resemblance with chymotrypsin, although the rate of hydrolysis of *Agave* protease is much lower.

Introduction

It was found that the extract of leaves of the plant *Agave americana variagata* would hydrolyze certain proteins. This was established after an observa-

* This paper formed part of a PhD thesis submitted to the Rand Afrikaans University.

** Present address: Department of Biochemistry, University of the Orange Free State, P O Box 339, Bloemfontein 9300 (Republic of South Africa).

tion that the plant sap irritates human skin to a marked degree. *Agave americana* (Century plant) of the family *Agavaceae* is a large ever-green succulent with long rigid sword-shaped leaves. Plants of this family are used for the production of cisal fibers. It is not related to any of the other plants from which proteases (endopeptidases) (EC 3.4.-) have been obtained, like the French bean, wheat, sorghum, papaya or fig. The plant does not lend itself to an easy attack from external parasites or other agents and is very hardy, occurring even in very barren areas. An endopeptidase, one of the proteolytic enzymes detected in the plant, was isolated and studied with respect to some of its physical and chemical properties, as reported here. Whereas most endopeptidases hitherto described from plants are acid sulphhydryl proteases [1—8], *Agave* protease is an endopeptidase with no sulphhydryl group, and its enzymic properties are to some extent similar to those of chymotrypsin, a serine protease.

Materials and Methods

The enzyme was isolated from leaves of *Agave americana variegata* plants growing near Johannesburg.

All chemicals and biochemicals used were of analytical grade. For the determination of protease activity, Hammersten casein and hemoglobin (B.D.H.), bovine serum albumin (Seravac) and glucagon (Lilly Laboratories) were used. Other substrates used were *N*-benzyloxycarbonyl L-tyrosine-*p*-nitrophenyl ester (B.D.H.), *N*-acetyl L-tyrosine ethyl ester (Seravac), α-*N*-benzoyl-L-arginine amide (N.B.C.o.), α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (B.D.H.), *N*-carbobenzoxyglycyl-L-β-phenylalanine (B.D.H.), *N*-carbobenzoxyglycyl-L-leucine (Fluka), hippuryl-L-arginine (Sigma), alanyl-β-naphtylamide (Fluka, N.B.C.o.), leucine-*p*-nitroanilide (N.B.C.o.) and lysine-*p*-nitroanilide (Fluka). The peptide substrates glycylleucine, glycylglycylleucine, leucylglycylglycine (N.B.C.o.) and leucylglycylphenylalanine (Miles-Yeda) were also used for characterization purposes.

Purification procedures were carried out at 5—6° C, unless otherwise stated. Dialysis of enzyme solutions was conducted over 16—24 h with three to four buffer changes and either pH or conductivity of solutions were measured to ensure adequate dialysis. Distilled water was deionized for preparation of solutions, and for other purposes. Buffers were prepared according to Gomori [9].

The protein concentration was determined by the Biuret procedure [10], the Lowry procedure [11] or a procedure based on absorbancy measurements [12]. In all cases bovine serum albumin was used as a standard. The first two methods were used throughout the isolation procedure, whilst the other method was employed during characterization procedures.

The enzyme was routinely assayed by the method described by Walsh and Wilcox [13] at 25° C. The increase in absorbance at 400 nm was followed spectrophotometrically and *N*-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester (Cbz-Tyr-ONp) was used as substrate at a concentration of 0.005 mM in 0.03 M Tris · HCl buffer, pH 7.8, containing 12% (v/v) methanol.

Analytical polyacrylamide gel electrophoresis [14] was conducted at room temperature with a Pleuger Acrylophor apparatus using 7.5% polyacrylamide gels at pH 5.0, 7.0 and 8.6. Proteins were visualized after electrophoresis with

either amido black staining or an affinity staining procedure using Cbz-Tyr-ONp as affinity reagent. After electrophoresis, gels were incubated in pH 7.8 buffer containing 0.5 mM Cbz-Tyr-ONp at 30° C for 1 h, and the enzyme could be observed as a clear yellow band [15]. Electrophoresis was also carried out under denaturing conditions in the presence of 7 M urea, 3 M guanidinium hydrochloride and 1% sodium lauryl sulphate respectively.

Isoelectric focussing was done for a period of 72 h using 1% (w/v) ampholyte concentrations over the pH ranges 3—10 and 5—8 at 5° C [16]. The pH, protein concentration (A_{280}) and enzymic activity of the collected fractions were determined.

For gel filtration studies Sephadex G-200 was used [17,18]. The gel column (2.5 × 90 cm) was calibrated with aldolase, yeast alcohol dehydrogenase, ribonuclease, cytochrome *c* (Boehringer); bovine serum albumin (Seravac); egg albumin (B.D.H.); trypsin (Pentex); and blue dextran (Pharmacia).

For ultracentrifugation experiments, a Beckman Model E analytical ultracentrifuge was used. Sedimentation studies were conducted at 56 000 rev./min at about 20° C, and diffusion studies at 9 000 rev./min at 5° C, in 12-mm pathlength standard type cells with aluminium centrepiece and sapphire windows. Boundary formation was observed with the ultra-violet optical system at 270 nm [19—21].

Amino acid composition was determined on a Beckman Model 118 amino acid analyzer after hydrolyzing quantities of 1.5 mg enzyme (accurately weighed) with 6 M HCl. Corrections were made for losses and quantitative recoveries of specific amino acids using different hydrolysis periods and conditions [22,23]. Tryptophan was determined by absorbancy methods [24,25]. Protein samples were dried under vacuum for 48 h over calcium chloride at room temperature prior to hydrolysis.

The sulphhydryl content of the enzyme was determined with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), under denaturing conditions [26] and the carbohydrate content of the enzyme by the anthrone method [27].

The kinetic studies were conducted with Cbz-Tyr-ONp as substrate in 0.03 M Tris · HCl buffer, pH 8.0, containing 12% (v/v) methanol. The final assay volume was 1 ml, 0.004 mg enzyme was used, and the concentration of Cbz-Tyr-ONp was varied from 0.002175 to 0.0435 mM at 25° C. Incubation procedures in 0.05 M sodium-phosphate buffer, pH 7.0, were also conducted with denatured proteins as substrates, 0.5% casein, bovine serum albumin and hemoglobin were used. The reactions were terminated with 5% trichloroacetic acid (final concentration) and absorbance measurements at 280 nm or Biuret determinations after neutralization served as control of the degree of hydrolysis. In all cases, also with Cbz-Tyr-ONp as substrate, corrections were made for background reactions.

The effect of pH variation was determined over the range pH 5—10 with the following substrates: denatured casein (45° C), Cbz-Tyr-ONp and *N*-acetyl-L-tyrosine ethyl ester (NAc-Tyr-OEt) [28] (25° C). The buffers used are indicated together with the results.

The effects of metal ions and other compounds were evaluated using the assay procedures mentioned. The compounds and concentration ranges tested will be mentioned together with the results.

The primary specificity of *Agave* protease was determined by incubation procedures with 0.5% casein as substrate in 0.05 M sodium-phosphate buffer, pH 7.8. Purified protease (0.02 mg/assay) was used during 1 h incubations at 30°C. Trichloroacetic acid (final concentration 5%, w/v) was added to the mixtures after incubation, and denatured proteins removed by centrifugation after a further 30 min at 30°C. A control was similarly prepared by adding the trichloroacetic acid before the 1 h incubation period. The clear supernatant fluids were fractionated separately on a Biogel-P2 column using deionized water, and the different peptide fractions were freeze-dried before further characterization. Amino-terminal amino acids of these peptide fractions were determined with dinitrofluorobenzene [29,30] and carboxyl-terminal amino acids with a mixed carboxypeptidase assay procedure [31].

Results

Purification of enzyme

Step 1: prepration of crude extract. 3 kg of leaves were finely cut and homogenized in a meat grinder with addition of NaCl to a final concentration of 0.15 M. The suspension was stirred for 1 h and pressed through a fine nylon cloth. Rubber gloves were worn to protect the skin. The 2000 ml plant extract was centrifuged at 10 000 × *g* for 20 min and the pH of the supernatant fluid was adjusted to pH 6.5. After dialysis of a fraction of the crude extract against deionized water for 16 h, the protein concentration [10,11] as well as enzymic activity were determined. This extract contained 48.7 units * protease and 65 g protein.

Step 2: ammonium sulphate fractionation. The dialyzed extract was fractionated with ammonium sulphate at pH 6.5 at a saturation level of 80% [32]. This mixture was left for 2 h and centrifuged at 12 000 × *g* for 30 min. The protein precipitate was dissolved in a minimum volume of 0.01 M Tris · HCl buffer, pH 7.2, and dialyzed against this buffer for 24 h. The dialyzed protein solution (400 ml), contained 20.16 g protein [10,11] and 44.4 units protease. The results of this step as well as all following steps are summarized in Table I.

Step 3: ethanol fractionation. The dialyzed protein solution was cooled to 0°C. Fractionation was carried out in an alcohol bath at −35°C with 95% ethanol precooled to −20°C. To the 400-ml solution, 200 ml ethanol were added slowly with constant stirring, whilst the temperature was kept below −8°C. This mixture was centrifuged at 5000 × *g* for 15 min in a precooled rotor and centrifuge, and the supernatant solution was directly used for further fractionation. The same procedure was used, but a volume of 400 ml ethanol was added. After centrifugation, the protein precipitates were immediately dissolved in deionized water and dialyzed for 16 h against deionized water. If the volume of dialysate was not too large, dialysis was continued against 0.02 M Tris · HCl buffer, pH 8.0, for 16 h, or otherwise the protein solution was freeze-dried before dialysis. This protein solution contained 1.805 g protein [10,11] and 39.7 units protease in a volume of 95 ml.

* A unit of enzyme activity is defined as the amount of enzyme that converts 1 μmol substrate to products in 1 min.

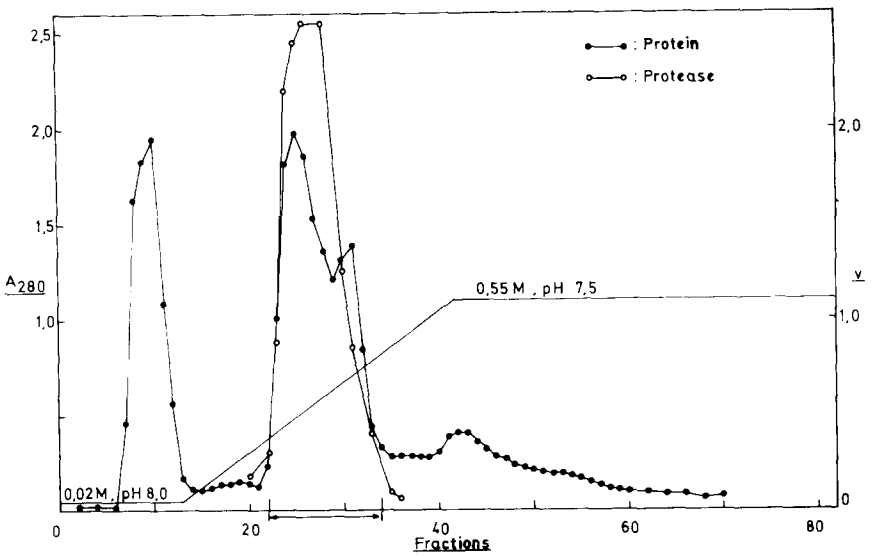

Fig. 1. Chromatography of *Agave* protease on a 2.5 × 50 cm DEAE-Sephadex column. A linear gradient, 0.02 M, pH 8.0—0.55 M, pH 7.5 was used, as indicated. The sample was obtained from step 3 and 12-ml fractions as indicated (1⟷1) were combined and used for further purification. Flow rate was 18 ml/h, A_{280} is the absorbance at 280 nm and v is the protease activity, μmol Cbz-Tyr-ONp hydrolyzed/min per ml.

Step 4: chromatography on DEAE-Sephadex. A DEAE-Sephadex A-25 column (2.5 × 50 cm) was prepared and equilibrated with 0.02 M Tris · HCl buffer, pH 8.0. The positions of the eluted proteins and the enzyme were determined by absorbancy measurements at 280 nm and enzyme activity measurements for this and all following chromatography steps. Information and results regarding this step are depicted in Fig. 1. The fractions containing the enzyme were combined, dialyzed against deionized water for 16 h and freeze-dried. This fraction contained 700 mg protein [10,11] and 33.6 units protease.

Step 5: chromatography on CM-Sephadex. The protein from step 4 was dissolved in 0.02 M sodium-citrate buffer, pH 5.2, dialyzed for 16 h against this buffer and centrifuged at 40 000 × g for 15 min. This fraction, containing 640 mg protein [10,11] and 30 units enzyme in a volume of 18 ml, was fractionated on the column. The results are depicted in Fig. 2.

More than one protein fraction displayed protease activity. These results were observed several times although the observed elution pattern did differ to some extent between different procedures. The fractions containing most of the protease were combined and contained 106 mg protein [10,11] and 23.5 units enzyme. After dialysis against deionized water for 16 h, the solution was freeze-dried. Gel electrophoresis (pH 8.6) displayed one major protein fraction although contaminants were still present in very low concentrations.

Step 6: chromatography on Sephadex G-200. The dry protein from step 5 was dissolved in 0.05 M sodium-phosphate buffer, pH 6.8, and dialyzed against this buffer for 16 h. Results of this step are shown in Fig. 3. The fractions containing the enzyme were combined, dialyzed against deionized water for 16 h and freeze-dried. This sample contained 55 mg protein [11] and 20.1 units pro-

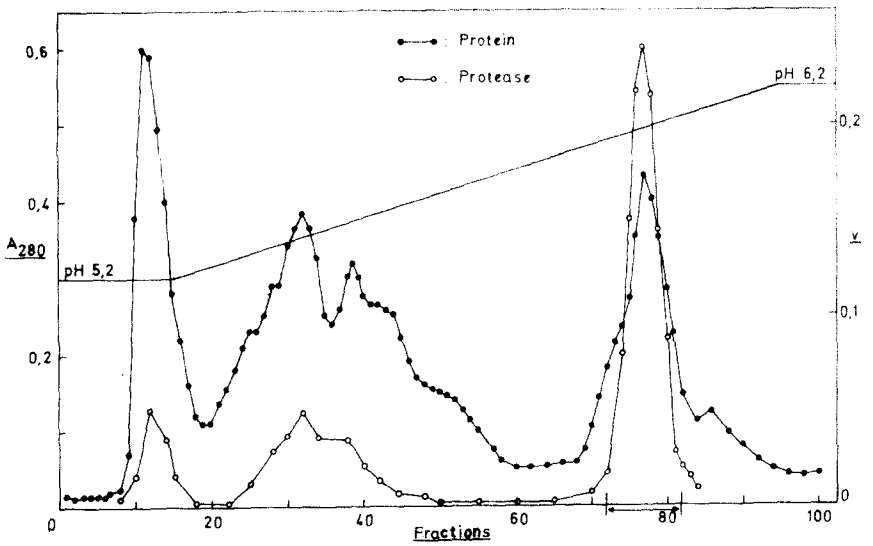

Fig. 2. Chromatography of *Agave* protease on a 2.5 X 39 cm CM-Sephadex column. A linear gradient, 0.02 M, pH 5.2 — 0.02 M, pH 6.2, was used. Sample from step 4, fractions of 6 ml each were collected, and combined as indicated (1⟷1). A_{280} is the absorbance at 280 nm, and v represents the μmol Cbz-Tyr-ONp hydrolyzed/min per ml.

tease. Analytical gel electrophoresis usually displayed only one fraction at this stage of the purification procedure.

Step 7: gel chromatography on Sephadex G-75. The protein sample was prepared as described in Step 6 and the results of this step are depicted in Fig. 4.

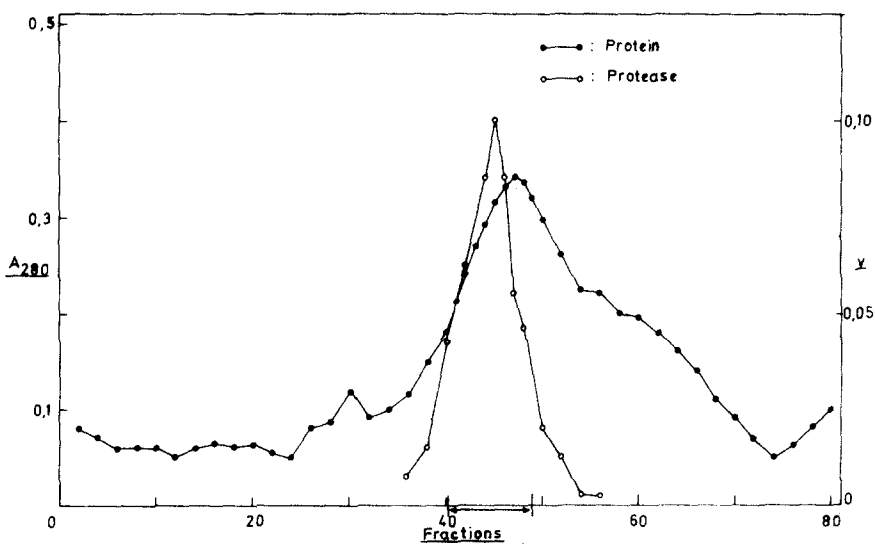

Fig. 3. Chromatography of *Agave* protease on a 2.5 X 90 cm Sephadex G-200 column. Sample from step 5. Buffer used was 0.05 M sodium-phosphate, pH 6.8, temperature 15—20°C, flow rate 16—19 ml/h, fractions of 6 ml each were collected and combined as indicated (1⟷1) for further purification. A_{280} is the absorbance at 280 nm and v is the protease activity, μmol Cbz-Tyr-ONp hydrolyzed/min per ml.

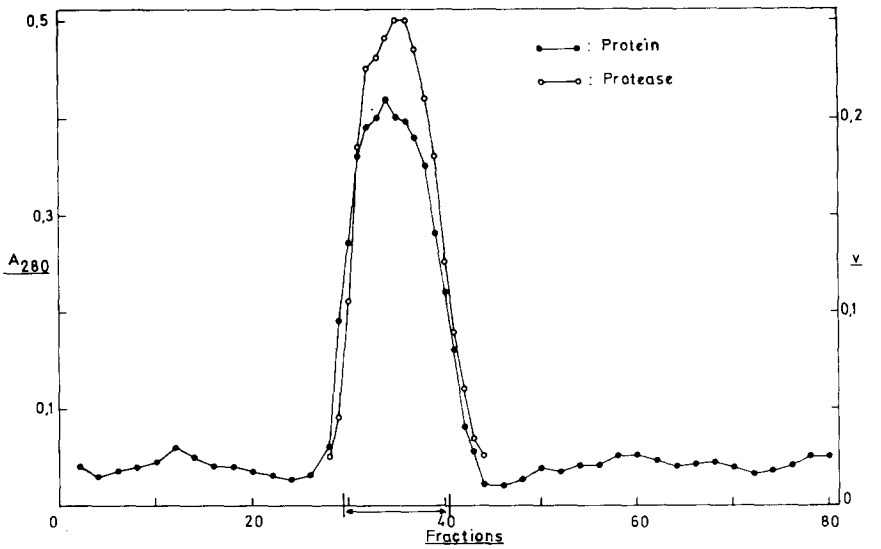

Fig. 4. Chromatography of *Agave* protease on a 2.5 × 90 cm Sephadex G-75 column. Sample from step 6. Buffer used was 0.05 M sodium-phosphate, pH 6.8, the flow rate was 16—18 ml/h, the temperature 5°C and the 6 ml fractions collected were combined as indicated (1⟷1) for further studies. A_{280} is the absorbance at 280 nm and v is the protease activity, μmol Cbz-Tyr-ONp hydrolyzed/min per ml.

The finally purified protease fraction contained 19.3 units enzyme and 43.8 mg protein [11]. The enzyme was purified 565-fold with a yield of 39.5%. The specific activity of the enzyme was 0.44 units/mg.

The results of the full isolation procedure are summarized in Table I and repeated procedures resulted in the same overall picture. According to analytical

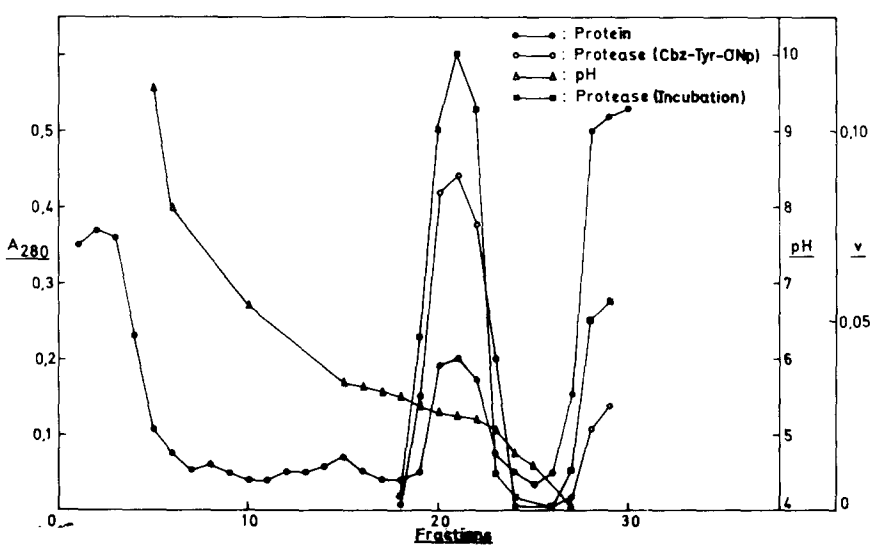

Fig. 5. Isoelectric focussing of *Agave* protease. 1% carrier ampholyte, pH 5—8, focussing for 72 h at 5°C. A_{280} is absorbance at 280 nm and v is the protease activity, μmol Cbz-Tyr-ONp hydrolyzed/min per ml whilst protease activity determined with the incubation procedure is indicated in arbitrary units.

TABLE I

RESULTS OF THE PURIFICATION PROCEDURE FOR OBTAINING THE PROTEASE ENZYME FROM *AGAVE AMERICANA VARIEGATA*

Step	Description	Volume (ml)	Total protein (mg)	Total enzyme (units)	Specific activity (units/mg Prot.)	Purification (fold)	Yield (%)
1	Cryde extract	2 000	62 500	48.7	0.000778	1	100
2	0—80% $(NH_4)_2SO_4$ precipitate	400	20 160	44.4	0.0022	2.83	91.0
3	0.5—1.5, vol EtOH precipitate	95	1 805	39.7	0.022	28.25	81.5
4	DEAE-Sephadex A-25 eluate	140	700	33.6	0.048	61.60	69.0
5	CM-Sephadex C-50H eluate	66	106	23.5	0.222	286.00	48.2
6	Sephadex G-200 eluate	72	55	20.1	0.365	470.00	41.2
7	Sephadex G-75 eluate	60	43.8	19.3	0.440	565.00	39.5

gel electrophoresis and other characterization procedures, the enzyme was obtained in homogenous form.

Properties of the enzyme

Electrophoretic homogeneity. Under the conditions of analytical polyacrylamide gel electrophoresis as used, (160 V, 5 mA/tube for 1 h) the enzyme migrated as a single protein band as observed by staining with amido black or an affinity staining procedure. The enzyme migrated about 11 mm under these conditions and was electrophoretically homogeneous. Preparative gel electrophoresis (results not shown) verified these results. Electrophoresis under a variety of denaturing conditions (7 M urea, 3 M guanidinium hydrochloride, 1% sodium lauryl sulphate) revealed no subunit structure for the enzyme.

Isoelectric point. The isoelectric point of *Agave* protease was 5.25 as determined in two different 1% ampholyte gradients. A distribution pattern as obtained is shown in Fig. 5.

Sedimentation coefficient. A corrected $S_{20,w}$-value of 4.37 S was obtained for *Agave* protease. This value is the average of determinations at concentrations varying from 0.55 to 0.74 mg/ml. Buffer concentrations were varied from 0.01 M to 0.17 M during these studies and no concentration dependence was observed.

Diffusion coefficient. The corrected diffusion coefficient of the protease as determined on the ultracentrifuge was $6.8 \cdot 10^{-7}$—$7.0 \cdot 10^{-7} cm^2 s^{-1}$. The value as determined by gel filtration studies according to the procedure of Andrews [18] was $6.6 \cdot 10^{-7} cm^2 s^{-1}$. The standards used were as indicated previously.

Other physical constants. By using the data obtained during gel filtration

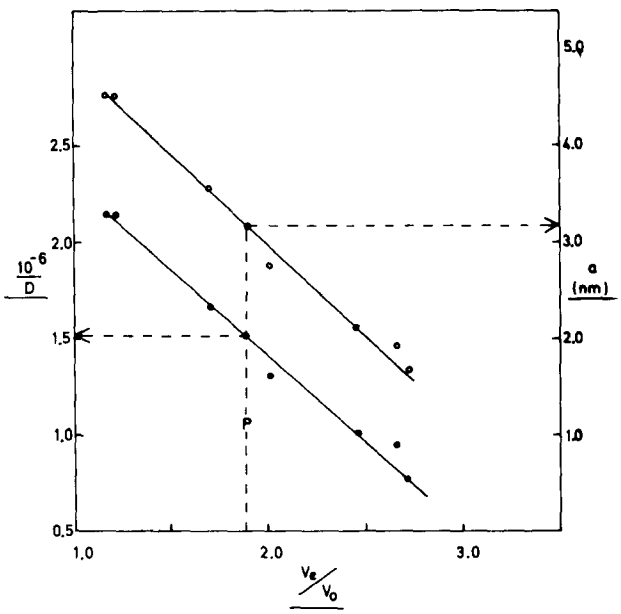

Fig. 6. Gel filtration studies with Sephadex G-200. Determination of the diffusion coefficient and the Stokes radius of the enzyme. Standards used are as indicated in Fig. 7.

studies [18], various physical constants were determined, as shown in Fig. 6. The enzyme had a Stokes radius (molecular size) of 3.18 nm and a frictional ratio, f/f_0 of 1.25. This last value indicated that the protease is a globular protein. The partial specific volume of the enzyme as determined from the amino acid composition [33] was 0.735 ml/g.

Molecular weight. By using the data concerning sedimentation and diffusion coefficients, and partial specific volume, the molecular weight of the enzyme was calculated to be 57 000. With gel filtration studies a value of 55 000 was found, as shown in Fig. 7.

Amino acid composition. The amino acid composition of the enzyme is indicated in Table II. The enzyme contains no cysteine, an observation also substantiated by repeated determinations of free sulphhydryl groups with Ellman's reagent under denaturing conditions.

Other chemical properties. Agave protease contained 8—10% carbohydrates on a weight basis as determined by the anthrone method. The carbohydrate fraction was not characterized further. Reaction of the enzyme with Ellman's reagent under various conditions indicated no free sulphhydryl groups, which is in accordance with the amino acid composition results.

Absorbance properties of the protease. The absorbance spectrum of the en-

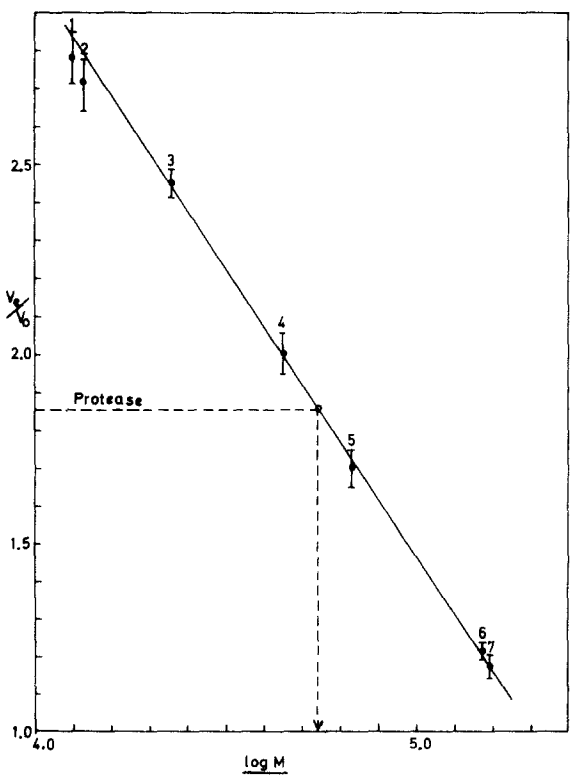

Fig. 7. Gel filtration studies with Sephadex G-200. Determination of the molecular weight of *Agave* protease. Standards used: 1, cytochrome *c*; 2, ribonuclease; 3, α-chymotrypsin; 4, egg albumin; 5, bovine serum albumin; 6, aldolase; and 7, yeast alcohol dehydrogenase. Conditions were as indicated in the text.

TABLE II

AMINO ACID COMPOSITION OF *AGAVE* PROTEASE

Amino acid	Calculated number of residues/mol protein	Number of amino acid residues/mol protein (Values rounded off)	Number of amino acid residues/100 residues
Lysine	22.00	22	5.44
Histidine	7.33	7	1.81
Arginine	8.66	9	2.14
Aspartic acid	45.33	45	11.22
Threonine	26.67	27	6.60
Serine	30.66	31	7.59
Glutamic acid	33.33	33	8.25
Proline	22.67	23	5.61
Glycine	36.67	37	9.07
Alanine	36.67	37	9.07
½ cystine	0	0	0
Valine	32.67	33	8.08
Methionine	4.00	4	0.99
Isoleucine	20.67	21	5.12
Leucine	34.67	35	8.58
Thyrosine	17.33	17	4.29
Phenylalanine	18.00	18	4.45
Tryptophan	6.80	7	1.68
Total	404.13	406	99.99

zyme in the ultraviolet region displayed a maximum value at 287 nm and a very slight shoulder at 292 nm. The enzyme had a molar absorbance index of $5.773 \cdot 10^4$ at 280 nm and a $E^{1\%}_{1cm}$-value of 9.621 at 280 nm, as based on accurately weighed vacuum-dried samples purified to a constant specific activity.

The physical and other properties of *Agave* protease are summarized in Table III.

General kinetic properties. Agave protease could hydrolyze a variety of protein substrates. The highest degree of hydrolysis was obtained with casein. The degree of hydrolysis of casein, hemoglobin and bovine serum albumin under identical conditions gave a ratio of 1 : 0.73 : 0.58 as based on the number of

TABLE III

PHYSICAL CONSTANTS OF *AGAVE* PROTEASE

Physical constant	*Agave* protease
$S_{20,w}$ (s^{-13})	4.37
$D_{20,w}$ ($cm^2\ s^{-1}$)	$6.8 \cdot 10^{-7}$—$7.0 \cdot 10^{-7}$
M_R (daltons)	$5.7 \cdot 10^4$
Stokes radius (nm)	3.18
f/fo	1.25
$\bar{v}$ (ml/g)	0.735
$E^{1\%}_{1cm}$	9.621
Molar absorbance index at 280 nm	$5.773 \cdot 10^4$
Carbohydrate content (%)	8—10
Isoelectric point	5.25

peptide bonds broken with the different substrates. By determining the amount of peptides or amino acids in solution with the Biuret method, after neutralization, it was observed that 3.84 mg casein were hydrolyzed per mg enzyme over a period of 30 min at 45° C.

The results of the kinetic studies with Cbz-Tyr-ONp as substrate are shown in Fig. 8. The apparent Michaelis constant of the enzyme for the substrate was $0.0345 \cdot 10^{-3}$ M and the V-value was 1.14 μmol substrate converted to products/min per mg enzyme, in other words, 1.24 mol substrate/mol enzyme per s. Lines were fitted to experimental data by the least squares method, and the results of repeated experiments were taken into account.

Effect of pH variation. The effect of pH variation on *Agave* protease activity using denatured casein as substrate, is shown in Fig. 9A. The activity of the enzyme is expressed as the increase in absorbance at 280 nm of the centrifuged trichloroacetic acid solution after an incubation period of 60 min at 45° C. The optimum pH for proteolysis was about pH 8.

The effect of pH variation, using the substrates Ac-Tyr-OEt and Cbz-Tyr-ONp, is shown in Figs. 9A and 9B. In both cases the optimum pH was between 7.8 and 8.0.

Agave protease was characterized further by conducting kinetic studies at different pH values [34]. By plotting log v and log V against pH, the apparent dissociation constants of the groups on the enzyme taking part in the catalytic process could be calculated, as these values moved through a maximum. These values were $pK_a \simeq 8.2$ and $pK_b \simeq 7.4$. It was also found that $pK_a = pK'_a$ and $pK_b = pK'_b$. The values of these constant as determined using Ac-Tyr-OEt as substrate, were similar to these reported here. These reported values are very similar to the values reported for chymotrypsin [35]. The apparent Michaelis constant of the enzyme was not influenced by variations of pH. According to

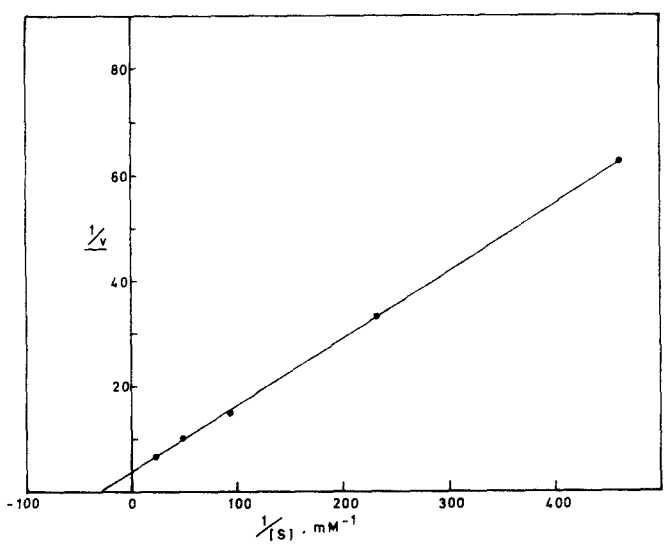

Fig. 8. Kinetic properties of *Agave* protease. Determination of the kinetic constants with Cbz-Tyr-ONp as substrate in 0.03 M Tris · HCl buffer, pH 7.8, containing 12% (v/v) methanol.

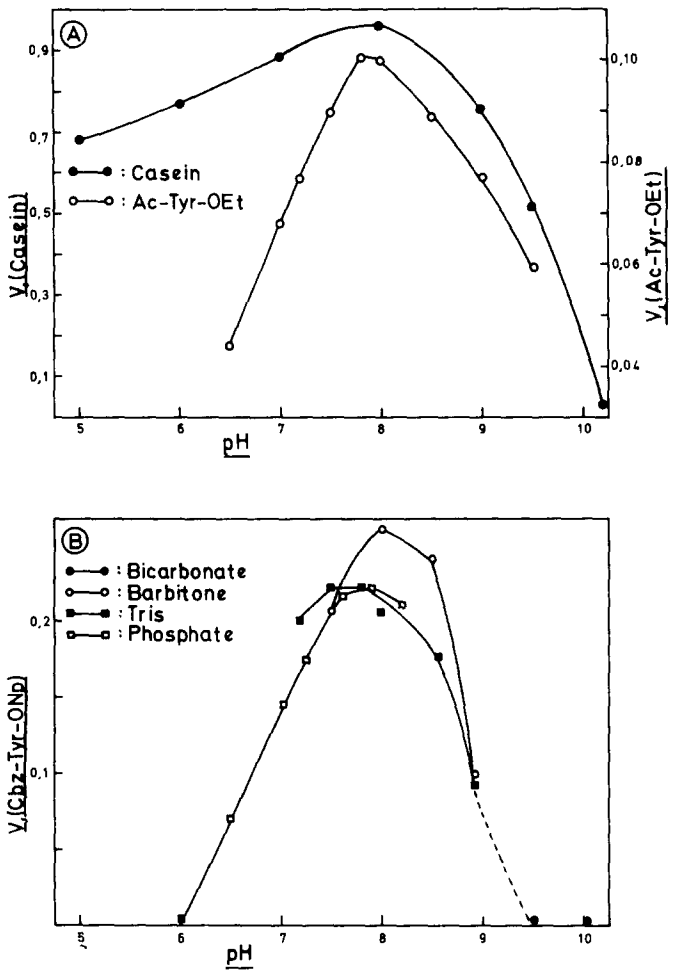

Fig. 9. Effect of pH variation on *Agave* protease. In A the optimum pH with denatured casein and Ac-Tyr-OEt as substrates are given, whilst in B the optimum pH with Cbz-Tyr-ONp as substrate, using different buffers is indicated. Conditions were as described in the text. v, protease activity, μmol substrate hydrolyzed/min per ml, except for casein where v is the increase in absorbance at 280 nm after 60 min at 45° C.

these results, this enzyme falls within group 4 as sub-divided by Laidler [34], i.e. both an acidic and a basic group on the enzyme take part in the catalysis, but the stubstrate as such does not bind to either of these groups.

Effects of metal ions and other compounds. Using denatured proteins as substrates the effects of a variety of compounds were determined. Only $HgCl_2$ (0.01 M, 84% inhibition) and $CuSO_4$ (0.02 M, 42% inhibition) inhibited the enzyme. EDTA (up to 0.03 M) had no effect on the enzyme, nor did NaCl or KCl, up to a concentration of 0.05 M.

Similar studies were conducted using Cbz-Tyr-ONp as substrate. These results are summarized in Fig. 10. The monovalent cations Na^+ and K^+ had no appreciable effect on the catalytic activity of the enzyme, and kinetic studies in

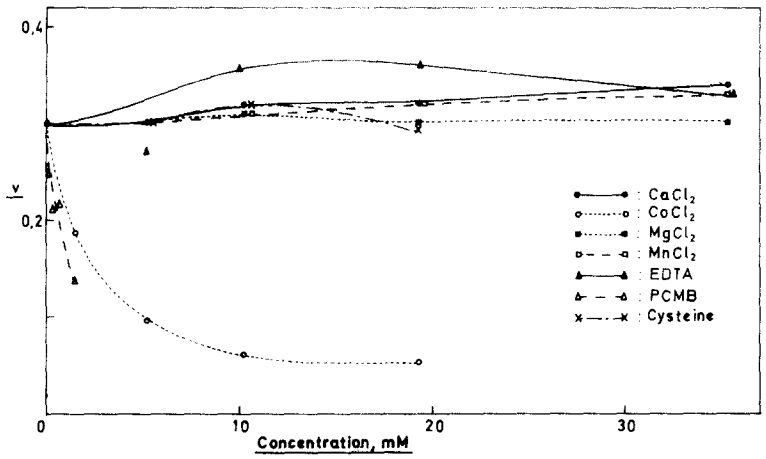

Fig. 10. Effects of metal ions, EDTA and the other indicated compounds on *Agave* protease. *v*, protease activity, in μmol Cbz-Tyr-ONp hydrolyzed/min per mg protein.

buffers of varying ionic strength also suggested that the catalytic constants are not affected by an increase in ionic strength.

It was found that *Agave* protease was inhibited by TosPheCH$_2$Cl. At a concentration of 0.2 mM the enzyme was inhibited 12.5%, whilst 50% inhibition was observed at a concentration of 0.5 mM TosPheCH$_2$Cl. This inhibition by TosPheCH$_2$Cl could have been mediated via either an effect upon serine or via the alkylation of a histidine residue. In this respect *Agave* protease resembles the serine proteases.

Specificity studies. These studies were carried out as described earlier. The determination of amino terminal end groups [29,30] had no conclusive results. There were no significant differences between the control and the incubation mixtures. Although the formed amino terminal groups did differ to some extent, there was no specific pattern in the appearance of newly formed amino-terminal amino acids. There were however clear differences in the carboxyl terminal amino acids [31] of the peptide residues present in the two different samples, in other words, the enzyme hydrolyzed the substrate at specific points as dictated by the amino acid supplying the carbonyl group of the peptide bond. These results were obtained several times under varying conditions. Repeated experiments were conducted to ensure that these amino acids could be identified to a high degree of accuracy. Amino acids found present at the carboxyl end of peptide residues, formed in large amounts during incubation, were tyrosine, pehnylalanine, leucine and/or isoleucine. All the generally occurring amino acids were used as references.

These results were also substantiated by results of studies with synthetic substrates. The substrates Cbz-Tyr-ONp and Ac-Tyr-OEt were both good substrates for the enzyme, whilst derivatives of arginine, Bz-Arg-OEt, Bz-Arg-NH$_2$, and Bz-Arg-NAn, could not be utilized by Agave protease. The dipeptide derivatives, Cbz-glycylphenylalanine, Cbz-glyccylleucine and hippuryl-arginine were not hydrolyzed by the enzyme. *Agave* protease displayed no aminopeptidase, carboxypeptidase or dipeptidase activities as determined with substrates under

suitable conditions for the determination of these and other mentioned activities [37]. In all these studies, substrate concentrations were varied in the concentration ranges suitable for *Agave* protease.

Discussion

The protease isolated from *Agave americana variegata* differed from most other proteases obtained from plants.

Although more than one active enzyme fraction were observed at certain stages, isolation of the enzyme did not prove to be too difficult. The presence of other proteolytic enzymes in the plant extract obliged us to carry out quite a number of fractionating procedures. The isolation procedure was repeated several times to obtain adequate quantities of the enzyme for the different studies. The enzyme was relatively stable and could be stored for considerable periods of time in dry form in the cold.

The protease was obtained in homogenous form according to purification to a constant specific activity, as well as other homogeneity determinations. The enzyme was homogenous with respect to gel electrophoresis, isoelectric focussing, gel filtration and ultracentrifugal analysis. All the physical constants of the enzyme, as well as other physical properties, were determined by repeated experiments and the average values of these different studies are indicated in Tables II and III.

However, a certain fraction of *Agave* protease, obtained after chromatography on CM-Sephadex, did display homogeneity with respect to molecular size, although this fraction was not electrophoretically pure. This phenomenon was not investigated further, as this fraction was not the one routinely used for further purification.

According to observations with the different protein substrates as well as the different synthetic substrates, *Agave* protease had a limited specificity. The enzyme displayed a marked esterolytic activity as indicated by the fact that Cbz-Tyr-ONp and Ac-Tyr-OEt were good substrates for the enzyme.

The optimum pH of the enzyme was always within the range pH 7.8—8.0, when using protein substrates or other suitable amino acid derivatives, and even using different buffers of varying ionic strengths. Kinetic studies at different pH values indicated a similarity between *Agave* protease and chymotrypsin. Considering the optimum pH of this protease, as well as the fact that EDTA did not influence the activity of the enzyme, *Agave* protease should be classified as an alkaline protease [36]. The enzyme thus differs from the plant proteases generally known, which are all sulphhydryl proteases, and also from chymotrypsin, an enzyme which is optimally active in the presence of metal ions, although the well-known inhibitor of serine proteases, $TosPheCH_2Cl$ did inhibit the *Agave* enzyme. *Agave* protease resembles the alkaline proteases from microorganisms and chymotrypsin, but seems to be a rather primitive form of protease, not needing any specific cofactors; furthermore, it has a rather slow conversion rate with the substrates tested.

Agave protease seems to catalyze preferably the hydrolysis of peptide bonds adjacent to firstly, the aromatic amino acids and secondly, the branched aliphatic amino acids. The peptide bonds formed by the carbonyl groups of

these amino acids were hydrolyzed. Small substrates like dipeptides and other amino acid derivatives were not hydrolyzed by *Agave* protease. This can be due to the presence of charged groups on these molecules that interfere with the catalytic process of the enzyme. Although indications of the primary specificity of *Agave* protease were obtained, a full description of this enzyme's properties will only be possible after detailed studies with a large number of peptides and proteins of which the primary structures are known.

Agave protease, an alkaline protease, from *Agave americana variegata* displayed unique properties for a plant enzyme. It differs markedly from the sulphhydryl proteases, with some properties similar to those of proteases from micro-organisms and some similar to those of chymotrypsin. The slow rate of protein hydrolysis suggests that the function of the enzyme in the plant is coupled to normal anabolism-catabolism rather than to a defence function. Considering the specificity of the enzyme as well as its stability towards organic solvents, it is most probably the agent irritating human skin.

Acknowledgements

The author wishes to express his thanks to the Rand Afrikaans University as well as the South African Council for Scientific and Industrial Research for research grants and also to Professor J.C. Schabort, who acted as promotor during these studies.

References

1 Arnon, R. (1970) in Methods in Enzymology (Perlman, G.E. and Lorand, L., eds.) Vol. XIX, pp. 226—244, Academic Press, New York
2 Kunimitsu, D.K. and Yasunobu, K.T. (1970) in Methods in Enzymology (Perlmann, G.E. and Lorand, L., eds.) Vol. XIX, pp. 244—252, Academic Press, New York
3 Liener, I.E. and Friedenson, B. (1970) in Methods in Enzymology, (Perlmann, G.E. and Lorand, L. eds.) Vol. XIX, pp. 261—273, Academic Press, New York
4 Murachi, T. (1970) in Methods in Enzymology (Perlmann, G.E. and Lorand, L., eds.) Vol. XIX, pp. 273—284, Academic Press, New York
5 McDowall, M.A. (1970) Eur. J. Biochem. 14, 214—221
6 McDowall, M.A. (1973) Biochim. Biophys. Acta 293, 226—231
7 Chao, L.P. and Liener, I.E. (1967) Biochim. Biophys. Res. Commun. 27, 100—106
8 Toro-Goyco, E., Maretzki, A. and Matos, M.L. (1968) Arch. Biochem. Biophys. 126, 91—104
9 Gomori, G. (1955) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.) Vol. 1, pp. 138—146, Academic Press, New York
10 Gornall, A.G. Bardawill, C.J. and David, M.M. (1949) J. Biol. Chem. 177, 751—766
11 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
12 Warburg, O. and Christian, W. (as given by Layne, E.) (1955) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.) Vol. 1, pp. 138—146, Academic Press, New York
13 Walsh, K.A. and Wilcox, P.E. (1970) in Methods in Enzymology (Perlmann, G.E. and Lorand, L. eds.) Vol. XIX, pp. 31—41, Academic Press, New York
14 Gabriel, O. (1971) in Methods in Enzymology (Jakoby, W.B., ed.) Vol. XXII, pp. 565—578, Academic Press, New York
15 Du Toit, P.J. (1975) Submitted for publication, Tydskr. vir Natuurwet
16 Versterberg, O. (1971) in Methods in Enzymology (Jakoby, W.B., ed.) Vol. XXII, pp. 389—412, Academic Press, New York
17 Andrews, P. (1964) Biochem. J. 91, 222—233
18 Andrews, P. (1970) in Methods of Biochemical Analysis (Glick, D., ed.) Vol. 18, pp. 1—53, Interscience Publishers, John Wiley and Sons, New York
19 Svedberg, T. and Pederson, K.O. (1940) The Ultracentrifuge, of The international series of Monographs on physics, (Fowler, R.H. and Kapitza, P., eds.) Oxford Clarendon Press

20 Schachmann, H.K. (1959) Ultracentrifugation in Biochemistry Academic Press, New York
21 Chervenka, C.H. (1969) A manual of methods for the analytical ultracentrifuge, Beckman Instruments Inc.
22 Eveleigh, J.W. and Winter, G.D. (1970) in Protein Sequence Determination (Needleman, S.B. ed.) pp. 91—123, Chapman and Hall, London
23 Beckman Amino Acid Analyzer Instruction Manual (1971) in Models 118 and 119 Amino Acid Analyzer, Section 2, pp. 1—10, Beckman Instruments Inc
24 Bencze, W.L. and Schid, K. (1957) Anal. Chem. 29, 1193—1196
25 Du Toit, P.J. (1975) Tydskr. vir Natuurwet 15, 100—102
26 Scoffone, E. and Fontana, A. (1970) in Protein Sequence Determination (Needleman, S.B., ed.) pp. 199—201, Chapman and Hall, London
27 Chaykin, S. (1966) in Biochemistry Laboratory Techniques pp. 88—89, John Wiley and Sons Inc., New York
28 Wilcox, P.E. (1970) in Methods in Enzymology (Perlmann, G.E., and Lorand, L., eds.) Vol. XIX, pp. 64—112, Academic Press, New York
29 Rendina, G. (1971) in Experimental Methods in Modern Biochemistry pp. 91—97, W.B. Saunders Company, Philadelphia
30 Narita, K. (1970) in Protein Sequence Determination (Needleman, S.B., ed.) pp. 27—38, Chapman and Hall, London
31 Blackburn, S. (1970) in Protein Sequence Determination, Methods and Techniques, pp. 194—199, Marcell Dekker Inc., New York
32 Dixon, M. and Webb, E.C. (1964) in Enzymes, p. 40, Longmans, London
33 Schachman, H.K. (1957) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.), Vol. IV, pp. 32—103, Academic Press, New York
34 Laidler, K.J. (1958) in The Chemical Kinetics of Enzyme Action, pp. 117—162, Oxford Clarendon Press
35 Hess, G.P. (1971) in The Enzymes (Boyer, P.D., ed.) Vol. III, pp. 213—248, Academic Press, New York
36 Matsumura, Y., Minamiura, N., Fukumoto, J. and Yamamoto, T. (1971) Agr. Biol. Chem. 35, 975—982
37 Perlmann, G.E. and Lorand, L. (1970) in Methods in Enzymology Vol. XIX, Academic Press, New York