ISOLATION OF ACETYLPEPTIDE FROM ENZYMIC DIGESTS OF TMV-PROTEIN*

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It has been established that native tobacco mosaic virus (TMV)-protein contains no N-terminal amino acid[1,2], but that after treatment with hot trichloroacetic acid (TCA)[1,3,4,5], or with hydroxylamine[6] appreciable amounts of proline appear as N-terminus. SCHRAMM, BRAUNITZER and their collaborators claimed that proline occurs as a hidden N-terminal amino acid[5,6] and they reported the isolation of DNP-prolyl-isoleucylglutamic acid[7] and, later, of a much longer crystalline DNP-prolyl peptide as N-terminal peptide[8]. Furthermore, BRAUNITZER[6] reported evidence that the hidden N-terminal proline was linked by amide linkage to the side-chain carboxyl group of an aspartic acid residue, the peptide chain thus forming a loop structure with C-terminal threonine. However, while suggestive, the evidence for the loop structure or the β-aspartyl–proline bond was not definite.

The author, therefore, attempted to isolate the cyclic peptide from enzymic digests of TMV-protein in order to establish its existence and determine its structure. If the loop part were not attacked by proteolytic enzymes and if it contained no basic amino acid residue, it should be quite acidic in nature. All peptides, except the C-terminal one, produced by tryptic digestion may be expected to contain basic amino acids, either lysine or arginine, as C-terminus owing to the specificity of the enzyme. Therefore, chymotrypsin and pepsin were used. For the separation of the sought-for acidic peptide, ion-exchange chromatography was employed. With the exception of strongly acidic phosphoserine peptides and cysteic acid peptides, all peptides are adsorbed on sulfonic acid ion-exchange resins in the hydrogen form. However, a cyclic peptide carrying a carboxyl but no basic group should be washed out from such a column by water.

Actually an acidic peptide was eluted with water from a column of the hydrogen form of Dowex-50 to which chymotryptic or peptic digest of TMV-protein had been applied. This fraction was further purified by a Dowex-2 column (chloride form) and was identified as acetylseryltyrosine. It represented the main acidic component of chymotryptic digests. In the present paper the method of isolation and the chemical structure of the acetylated dipeptide will be described and its location in the original protein will be discussed.

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EXPERIMENTAL

TMV-protein

Both native and denatured TMV-proteins* were used. The former was prepared by the acetic acid method[9] and the latter by sodium dodecyl sulfate (SDS) treatment[1,10].

Enzymic digestion

The protein was digested by crystalline chymotrypsin (Worthington Biochemical Corp.) or pepsin (Worthington Biochemical Corp.) at room temperature. In both digestions, substrate concentration was 1% and the ratio of substrate to enzyme was 100:1. During digestion, the pH was maintained at 8.0 in chymotryptic and at 2.0 in peptic digestions, by the addition of dilute sodium hydroxide or hydrochloric acid. The SDS-treated protein required a higher pH for solution (pH 10), but remained in solution upon subsequent adjustment to pH 8.0. Both native and denatured proteins precipitated at the acidic pH used for peptic digestion. After 24 hours digestion, the pH of the hydrolysis mixtures was adjusted to 3.8 and insoluble peptide fragments were removed by centrifugation. In the SDS protein the average number of bonds split by chymotrypsin and pepsin, as determined by ninhydrin, were 14.1 and 22.0 per mole (mol.wt., 18,000) respectively, and the insoluble material removed at pH 3.8 averaged 44.3 and 34.8% by weight, respectively (Table I). Peptic digestion of the native protein, yielded no precipitate at pH 3.8.

Isolation of the acidic peptide

The soluble fraction of the enzymic digests was applied to Dowex-50 ($\times$ 2, hydrogen form, 50 $\sim$ 100 mesh) columns (1 $\times$ 20 cm) at a flow rate of about 0.5 ml per minute. To minimize the possibility that large peptides of the digests would pass through unadsorbed, low crosslinked resin was used[11]. After the sample solution had penetrated the resin the column was washed with water at the same flow rate as described above, and 5 ml aliquots of the effluent were collected automatically. The amount of peptide in each fraction was estimated by a modified Folin method[12] using 0.5 ml of each fraction. The acidic peptide appeared in fractions 2 to 10 after the hold-up volume. The peptide isolated by drying or lyophilization, was obtained in an average yield of 2.0% of the weight of the original protein (Table I). It was found that this fraction contained small amounts of several peptides besides the main component, judging from paper chromatograms developed with *n*-butanol acetic acid water (4:1:1, vol.). Further purification was achieved by means of a Dowex-2 ($\times$ 10, 200 $\sim$ 400 mesh) column in the chloride form using 0.015*N* hydrochloric acid as developer at a flow rate of 0.2 ml/min as shown in Figs. 1 and 2. Depending on the column dimensions (2 $\times$ 15 cm or 1 $\times$ 20 cm) 6- or 3-ml aliquots were collected. Peak C-2 (chymotryptic peptide) and peak P-4 (peptic peptide) were isolated with 81 and 69% yields as main components from the two acidic peptide mixtures, respectively.

In control experiments in which TMV-protein or enzyme alone was incubated under the same conditions as above, no Folin-positive material was eluted with water from a Dowex-50 column.

Amino acid sequence of the acidic peptide

About 0.2 mg of the peptide was hydrolyzed with 0.5 ml of constant boiling hydrochloric acid in a sealed tube at 100°C for 15 hours. The evaporated hydrolysate was treated with 1-fluoro-2,4-dinitrobenzene (FDNB) as usual[13], DNP-amino acids produced were separated and estimated by two-dimensional paper chromatography, as described by LEVY[14].

The C-terminal amino acid was characterized and estimated by both the hydrazinolysis[15] and carboxypeptidase[18] methods. In the hydrazinolysis method, isovaleraldehyde was used to separate the amino acid hydrazides from the C-terminal free amino acid, as suggested by AKABORI *et al.*[16]. Otherwise the procedure was as used by NIU AND FRAENKEL-CONRAT[17]. Carboxypeptidase experiments were performed at pH 7.8 with a substrate to enzyme ratio of 20:1. Liberated amino acids from the C-terminal position at various periods of time were converted to DNP-derivatives and estimated by paper chromatography as suggested by HARRIS[18].

N-terminal amino acid analysis was carried out by SANGER's FDNB method[13]. The DNP-peptide was hydrolyzed with constant boiling hydrochloric acid at 100°C for 8 hours in a sealed tube. As described later, the peptide did not contain any free N-terminal residue and it was found that the α-amino group of the peptide was masked by an acyl group. To characterize the acyl group, hydrazinolysis was again used. The evaporated hydrazinolysate, which was obtained by treating with anhydrous hydrazine under the usual conditions[17], was directly applied to paper and developed one-dimensionally with a pyridine–aniline water (9:1:4, vol.)[15] or collidine water mixture (10:2, vol.). The resulting acylhydrazide and amino acid hydrazides were detected on paper by the ammoniacal silver nitrate reagent[19].

Standard hydrazides were synthesized by heating esters of fatty acids with 1.5 moles of hydrazine in ethanol at 100°C for 1 hour. The solvent and excess hydrazine were evaporated in a

* Both protein preparations were kindly supplied by Dr. H. FRAENKEL-CONRAT.

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vacuum desiccator over conc. sulfuric acid at room temperature. The crystalline formyl and acetyl hydrazides had melting points of 48 ~ 50°C and 64 ~ 65°C, respectively, but propionyl hydrazide was not crystallized. In each case the yields were almost quantitative and the homogeneity of the hydrazides was confirmed by paper chromatography. Serine hydrazide was also synthesized by the reaction of serine ethylester and hydrazine in ethanol at 60°C for 1 hour. After evaporation of the solvent and excess hydrazine, only syrupy serine hydrazide was obtained. AKABORI *et al.*[16] previously succeeded in obtaining the crystalline compound.

TABLE I

YIELDS OF ACIDIC PEPTIDES IN THE ENZYMIC DIGESTS OF TMV-PROTEIN

Expt. no.	*Protein*	*Enzyme*	*Bonds split*	*Ppt. at pH 3.8*	*Yield of the acidic peptide**	
					*Folin color***	*Weight*
				%	%	%
1	SDS-treated	Chymotrypsin	13.9	—	2.5	2.0
2	SDS-treated	Chymotrypsin	12.5	—	2.3	2.3
3	SDS-treated	Chymotrypsin	11.7	25.5	2.4	2.1
4	SDS-treated	Chymotrypsin	16.3	54.6	2.3	1.6
5	SDS-treated	Chymotrypsin	15.9	38.0	3.7	2.1
6	SDS-treated	Chymotrypsin	14.3	49.0	3.1	1.9
7	Acetic acid-treated	Chymotrypsin	—	—	3.0	1.9
8	Acetic acid-treated	Chymotrypsin	—	—	2.6	—
9	Acetic acid-treated	Chymotrypsin	—	—	2.9	1.2
10	Acetic acid-treated	Chymotrypsin	—	—	2.3	1.7
11	Acetic acid-treated	Chymotrypsin	—	—	3.0	—
Average			14.1	44.3	2.7	1.9
12	SDS-treated	Pepsin	19.4	39.7	5.7	1.4
13	SDS-treated	Pepsin	24.6	29.8	3.5	1.1
14	Acetic acid-treated	Pepsin	—	—	4.9	3.2
15	Acetic acid-treated	Pepsin	—	—	3.4	1.7
16	Acetic acid-treated	Pepsin	—	—	3.2	2.7
Average			22.0	34.8	4.1	2.0

* Yield is based on protein used, including amount of precipitate resulted at pH 3.8.
** Known amounts of the chymotryptic digest of TMV protein were used for the preparation of the standard curve.

RESULTS

The essential homogeneity and the identity of the main peaks, C-2 and P-4 shown in Figs. 1 and 2 in the chymotryptic and in the peptic digests, respectively, were confirmed by paper chromatograms (R_F of both, 0.70 in *n*-butanol–acetic acid–water mixture). Their ultraviolet (UV) absorption curves were identical and their amino acid composition as determined by the FDNB method also proved to be the same. These peptides were negative to ninhydrin, and were detected on paper by the chlorine–starch–iodide reaction suggested by RYDON AND SMITH[20]. As shown in Fig. 3, the UV-absorption curve of the peptide indicated that it contained tyrosine and no other UV-absorbing materials. Constituent amino acids were only tyrosine and serine and the molar ratio of the former to the latter was 1:1.2. From the UV spectrum, the tyrosine content was calculated as 34% by weight. All the figures of amino acid analyses were calculated on the basis of tyrosine content as measured by the UV

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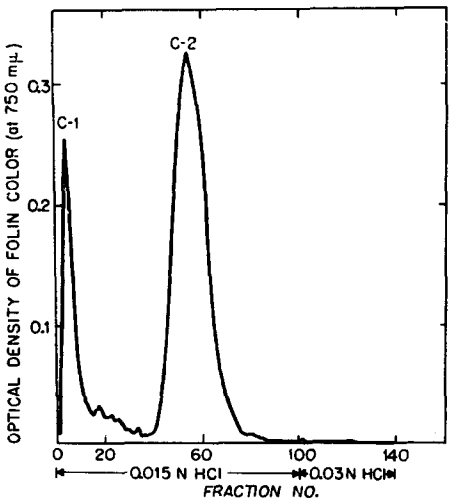

Fig. 1. Purification of acidic peptide from chymotryptic digest isolated by Dowex-50 column chromatography. Chromatography of 13.8 mg of the acidic peptide fraction was carried out on a 2.0 × 15-cm column of chloride form of Dowex-2-X10. The effluent was collected in 6-ml fractions. Peak C-2 corresponded to 81% of acidic peptide fraction applied.

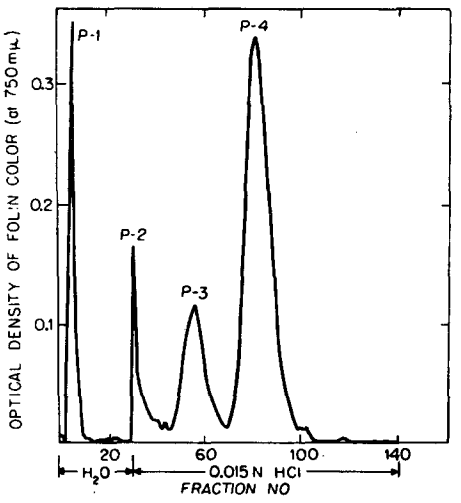

Fig. 2. Purification of acidic peptide from peptic digest isolated by Dowex-50 column chromatography. Chromatography of 5.7 mg of the acidic peptide fraction was carried out on a 1.0 × 21-cm column of chloride form of Dowex-2-X10. The effluent was collected in 3-ml fractions. Peak 3 and Peak 4 corresponded to 19 and 62% of acidic fraction applied. In this chromatography, initially, contaminated neutral peptides were washed out by water, then the development was performed by 0.015*N* hydrochloric acid.

spectrum. Corrected DNP-analyses indicated 0.81 and 0.69 mole of serine and tyrosine per mole of the peptide respectively. Preliminary results of ion-exchange chromatography have been 0.98 and 0.93 for these two amino acids.

C-terminal amino acid analyses showed that the tyrosine occurred at the end position. 0.77 mole per mole of the peptide appeared as free tyrosine after hydrazinolysis, and the expected amount of tyrosine was liberated by carboxypeptidase action after 22-hours digestion, at which period the digestion reached equilibrium. In both tests only traces of free serine were detected. Considering the composition of the peptide and the specificity of chymotrypsin this result could be expected. From these analyses it must be concluded that the peptide is a dipeptide and has the sequence: seryltyrosine. Since the observed tyrosine content (34% by weight) is only 59% of the theoretical, 41% of the material must be attributed to non-peptidic contaminants.

N-terminal analysis by the DNP-method gave 0.01 mole DNP-serine per mole peptide, *i.e.* essentially a negative result. Nevertheless, the peptide formed a DNP-derivative which was soluble in organic solvent, such as ether or ethylacetate, and which could be located in almost the same position as DNP-serine on the two-dimensional paper chromatogram, prepared according to LEVY[14]. This DNP-derivative of the peptide, however, had no color, and was detected on the paper by its strong absorption of UV light. The spot was eluted with 1% sodium bicarbonate, acidified, and the DNP-peptide extracted into ethylacetate. The peptide was dissolved in 1 N hydrochloric acid after removal of the solvent and its UV-absorption curve was plotted. As

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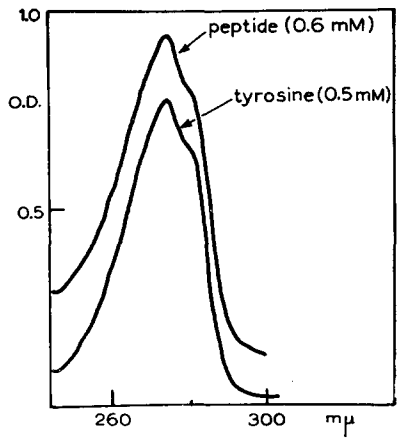

Fig. 3. UV absorption spectrum of acidic peptide and of tyrosine.

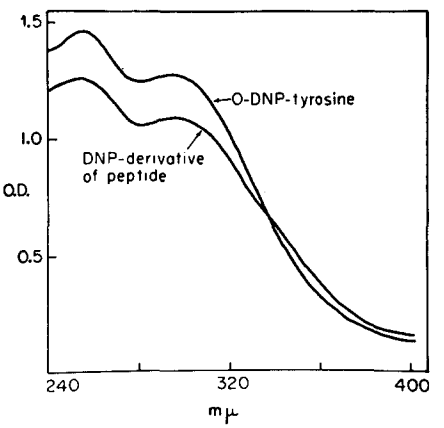

Fig. 4. UV absorption spectrum of DNP-derivative of acidic peptide and of O-DNP-tyrosine.

shown in Fig. 4, this curve agreed with that of O-DNP-tyrosine. If a DNP-group had been introduced on the α-amino group of the peptide, a peak near 360 mμ should appear, but no peak was observed around this wave length. Consequently it must be considered that the α-amino group is blocked. The findings that the peptide was negative to ninhydrin was compatible with above concept. Since qualitative tests for carbohydrate, phosphate and nucleic acid were negative, the possibility that the substituting group was a simple acyl group was then considered. Hydrazinolysis seemed to be a suitable method to characterize the acyl substituent as the acyl hydrazide by paper chromatography[19]. About 0.2 mg of peptide was treated with 0.2 ml of anhydrous hydrazine in a sealed tube at 100°C for 10 hours. After evaporation of excess hydrazine, the hydrazinolysis mixture was applied to paper. Descending paper chromatography was carried out with pyridine–aniline–water and collidine–water as solvent. In the former solvent, three distinct spots appeared by ammoniacal silver nitrate reagent as listed in Table II. These corresponded to hydrazine, serine hydrazide and acetyl hydrazide respectively. In the latter solvent system, an appreciable amount of an additional unidentified spot appeared besides the above three spots. The hydrazinolysate of an authentic sample of DL-seryl-DL-tyrosine* also gave serine hydrazide and the unknown spot and crude serine hydrazide contained the same unknown material. Therefore the unidentified spot might be derived from serine or serine hydrazide. The amount of acetyl hydrazide in the hydrazinolysate of the peptide was about one mole per mole peptide as judged from the color intensity of the spot compared to that of a known amount of synthetic acetyl hydrazide. Furthermore, a spot corresponding to ammonium acetate or formate was detected in the conc. ammonia hydrolysate of the peptide (at 100°C for 24 hours in a sealed tube) by paper chromatography[21] (for R_F values see Table III). The possibility of an ammonium formate spot, however, was ruled out by the observation of paper chromatograms of the hydrazinolysate described above. The amount of acetate in the 25% sulfuric acid hydrolysate of the peptide was estimated by CONWAY's diffusion method[22]. Only about 50% of standard acetate was recovered after 24 hours diffusion at 37°C. An average 1.1 and 0.97 moles acetate was

* DL-Seryl-DL-tyrosine used was prepared by Dr. J. I. HARRIS.

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TABLE II

R_F VALUES OF SEVERAL HYDRAZIDES*

Solvent: pyridine–aniline–water (9:1:4, vol.)

	Standard substance	*Hydrazinolysate of peptide*
Formyl hydrazide	0.58	—
Acetyl hydrazide	0.66	0.66
Propionyl hydrazide	0.75	—
Serine hydrazide	0.51	0.51
Hydrazine	0.39	0.40

Solvent: collidine–water (10:2, vol.)

	Standard substance	*Hydrazinolysate of peptide*	*Hydrazinolysate of* DL-*seryl*-DL-*tyrosine*
Formyl hydrazide	0.28	—	—
Acetyl hydrazide	0.35	0.33	—
Propionyl hydrazide	0.50	—	—
Serine hydrazide	0.074	0.073	0.074
Hydrazine	0.00	0.00	0.00
Unidentified spot		0.28	0.27

* Detecting reagent: Ammoniacal silver nitrate solution (0.1 *N* $AgNO_3$–5 *N* NH_4OH mixture, 1:1, vol.).

TABLE III

R_F VALUES OF SEVERAL LOWER FATTY ACID AMMONIUM SALTS*

Solvent: 95% Ethanol–conc. Ammonium hydrixide (99:1, vol.)

	Standard substance	*Hydrolysate of peptide*
Ammonium formate	0.26	—
Ammonium acetate	0.28	0.27
Ammonium propionate	0.34	—
Unknown	—	0.054

* Detecting reagent: 1% Bromcresol green solution in absolute ethanol.

found per mole of the peptide and of the SDS-treated protein hydrolysates, respectively, using this recovery factor. It thus appears probable that the acyl group is acetyl and the structure of the peptide is acetylseryltyrosine.

From the above structure, the molecular weight and the theoretical yield of the peptide were calculated as 310 and 1.7% respectively. The observed average yield of the acetylpeptide in chymotryptic digests was 1.6% by weight, but part of this material was non-peptidic and probably contributed by the exchange resin.

DISCUSSION

The original intent to isolate a cyclic peptide from the enzymic digest of TMV-protein was unsuccessful. Instead, an acetylated dipeptide, acetylseryltyrosine was obtained. Since no free N-terminal amino acid has ever been found in native or denatured TMV-protein[1,2] it appears possible that this acetyl peptide is located in the terminal position of the chain.

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Another possibility for the location of the acetyl peptide cannot be excluded, however. Since TMV-protein contains two lysine residues of which only one is reactive to FDNB[1], the acetylated peptide might be derived from a branch chain attached to an ε-lysine residue.

In view of this possibility, the present finding does not rule out the existence of a loop peptide as postulated by BRAUNITZER[6]. However, search for evidence that there occurs in TMV a particularly labile peptide linkage, such as the supposed proline–β-aspartyl linkage, has consistently given negative results. Analysis by hydrazinolysis of the protein prepared with boiling TCA has shown the appearance of C-terminal amino acids in amounts approximately equivalent to the N-terminal groups formed. While the latter is predominantly proline, the former consist of various residues in non-stoichiometric amounts. Unpublished experiments by Dr. L. K. RAMACHANDRAN also have failed to confirm the sequence of amino acids claimed to be adjacent to proline[7]. It appears from these studies that peptide bonds involving the imino group of proline are hydrolyzed in a random and non-stoichiometric manner by TCA treatment.

Thus, the nature of the N-terminal residue has remained completely indeterminate, and it appears most probable that this position is occupied by the acetylpeptide here described. The possibility must be considered, however, that the described peptide is produced through acyl shift. Thus if O-acetylseryltyrosine occurred within the protein and if it were liberated by the action of chymotrypsin, it is possible to imagine that the O-acetyl group might have shifted to N-acetyl in the alkaline medium* used during enzyme digestion. This possibility, however, could be excluded by the isolation of the same peptide from peptic digest of the protein, because no neutral or alkaline conditions were used throughout the entire procedures of peptic digestion and isolation. Therefore the N-acetylseryltyrosine must exist as such in the original virus protein.

The isolation of the present peptide probably represents the first demonstration of an acetyl peptide in a naturally occurring protein. The finding of this acetyl peptide seems to suggest that one must be cautious in assuming a cyclic structure for proteins lacking N-terminal amino acid residues**. Preliminary studies*** have yielded a similar result in the case of ovalbumin, where the N-terminal position was found substituted by the carbohydrate moiety.

ACKNOWLEDGEMENTS

The author wishes to express his appreciation to Drs. H. FRAENKEL-CONRAT AND L. K. RAMACHANDRAN for stimulation and discussion throughout this investigation.

* For example, D. F. ELLIOTT, *Biochem. J.*, 50 (1952) 542; P. EDMAN AND L. JOSEFSSON, *Nature*, 179 (1957) 1189.

** During the preparation of this manuscript, a paper by J. I. HARRIS AND A. B. LERNER, *Nature*, 179 (1957) 1346, came to the author's attention, in which amino acid sequence of α-melanocyte-stimulating hormone isolated from pig pituitary has been determined. They found that the α-amino group of the serine residue situated at the chain end was blocked by an undetermined substituent.

*** K. NARITA, F. OKAMOTO, M. KANNO AND N. MATSUDA, unpublished experiments. A carbohydrate-containing peptide in a peptic digest of ovalbumin was not eluted from a Dowex-50 column (hydrogen form) by washing with water. However, after acetylation and digestion, an acidic carbohydrate-containing peptide which has no N-terminal residue could be eluted. The yield of the peptide depended upon the extent of the acetylation. This peptide fraction was ninhydrin negative and contained mannose, glucosamine and about ten amino acid residues including lysine.

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SUMMARY

N-acetylseryltyrosine was isolated from the chymotryptic and peptic digests of TMV-protein. The structure of the acetylpeptide purified by ion-exchange chromatography, was studied by the FDNB method, hydrazinolysis and the use of carboxypeptidase. For the characterization of the acyl group in the peptide, hydrazinolysis was also used. The location of the acyl peptide in the protein was discussed.

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PURIFICATION OF GLUTAMIC-ASPARTIC TRANSAMINASE

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Several methods for the partial purification of glutamic-aspartic transaminase from heart muscle have been developed[1-4], the most recent being that by CAMMARATA AND COHEN in 1951. These purification schemes are based on the classical methods of fractional precipitation with salts and organic solvents. During the past few years, however, new and more effective techniques for the fractionation of proteins have been developed. The remarkable success with which they have been applied to the purification of other enzymes seemed to make it worth while to try a new approach

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