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The Primary Structure of Aspartate Aminotransferase from Pig Heart Muscle. Limited Peptic Digestion

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Chimica biologica. — *The Primary Structure of Aspartate Aminotransferase from Pig Heart Muscle. Limited Peptic Digestion* (*). Nota di FRANCESCA RIVA, DONATELLA BARRA, FRANCESCO BOSSA, MASSIMO CARLONI, PAOLO FASELLA, SHAWN DOONAN, HILARY J. DOONAN, ROBIN HANFORD e JOHN M. WALKER, presentata (**) dal Socio A. ROSSI-FANELLI.

RIASSUNTO. — I peptidi ottenuti mediante idrolisi peptica limitata dell'aspartico aminotransferasi sono stati purificati ed è stata determinata la loro sequenza in aminoacidi.

Sono stati prodotti in questa digestione frammenti che non sono comparsi nella precedente idrolisi peptica estensiva.

Questi frammenti hanno fornito utili « overlaps » con peptidi ottenuti digerendo l'aspartico amino transferasi con tripsina, termolisina, pepsina ed elastasi.

INTRODUCTION

The primary structure of cytoplasmic aspartate aminotransferase (L aspartate-2-oxoglutarate aminotransferase EC 2.6.1.1) from pig heart muscle has been determined [1], [2] and the results of digestion of the protein with trypsin thermolysin, elastase and pepsin have been published [3], [4], [5].

The present paper describes in detail results obtained from a peptic digestion, performed under different conditions from those already reported, the object of which was to restrict the extent of hydrolysis.

Pepsin was used in the early work, in spite of its very low specificity, because it digested aspartate aminotransferase to a completely soluble mixture of peptides. This was also true when milder condition of digestion were used but in this case we have been able to obtain longer fragments which did not appear in the previous digest. Some of these were of particular interest, providing useful overlaps with peptides obtained by digestion with other proteases.

Methods of purification included ion exchange chromatography, paper chromatography and high voltage paper electrophoresis.

(*) This research was carried out in the Istituto di Chimica Biologica e di Biochimica Applicata and Centro di Biologia Molecolare del Consiglio Nazionale delle Ricerche, Università di Roma, Italy and in the Christopher Ingold Laboratories, Dept. of Chemistry, University College, London.

(**) Nella seduta del 20 aprile 1974.

Materials and Methods

Aspartate aminotransferase was prepared by the method of Martinez-Carrion *et. al.* [6].

The carboxymethylated [7] lyophilized protein (1.2 gr) was suspended in 5% formic acid (300 ml) and digested with pepsin (1/100 *w/w* for 10′ at room temperature).

The peptide mixture was subjected to a preliminary fractionation by gel filtration through Sephadex G–25 equilibrated in acetic acid (10% *v/v*). The fractions obtained were further purified by ion exchange chromatography using SP–Sephadex C25. Gradients used were from pH 2.7 (0.05 M pyridine formate) to pH 6.5 (2M pyridine acetate) or from pH 3.1 (0.2 M pyridine formate) to pH 6.5 (2 M pyridine acetate) (fig. 1).

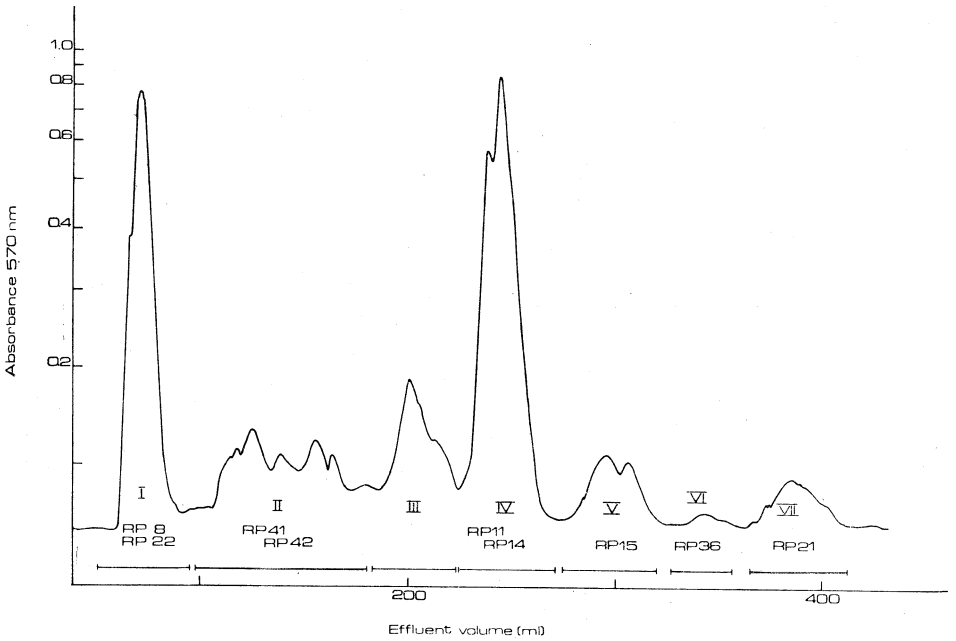


Fig. 1. – Elution profile from SP Sephadex C–25. The peptides were eluted with the following nine chambers gradient:

Chamber	0.2M pyr-formate pH 3.1	2M pyr-acetate pH 5.1	2M pyr-acetate pH 6.5	H_2O
1	216	—	—	—
2	216	—	—	—
3	216	—	—	—
4	216	—	—	—
5	132	36	—	48
6	12	108	—	90
7	—	208	—	—
8	—	156	52	—
9	—	—	208	—

and monitored by ninhydrin reaction after alcaline hydrolysis. Flow rate was 52 ml/h.

Further purification of fractions from ion exchange chromatography was obtained by paper chromatography and [8] high voltage paper electrophoresis in a liquid cooled tank system at pH 1.9 (formic acid-acetic acid-water 1 : 4 : 45 by vol).

The purity of peptides was established by the dansylation technique [9]. The technique of complete dansylation of the hydrolysate reported in our previous paper [3] was used to obtain qualitative amino acid compositions.

Quantitative analysis was carried out using a Bio Cal B 200 automatic analyzer (single column system) after hydrolysis of sample (25–50 nmol) as described by Sanger and Thompson [10].

C–terminal sequences were established by hydrolysis with carboxypeptidase C [11] followed by quantitative aminoacid analysis. Hydrolyses using aminopeptidase were performed as described by Light [12]. Sequence studies with the dansyl Edman technique were carried out by the method of Hartley [13] as previously described [3]. The presence of tryptophan in a peptide was indicated by a positive response to the Ehrlich reaction on paper and by the purple colour formed during cyclization and cleavage of the phenylthiocarbamoyl peptide in trifluoroacetic acid during sequence analysis until the step at which tryptophan was removed. Amide assignments were based on previous work.

RESULTS

From the total hydrolysate, 42 peptides were isolated which accounted for 67 % of the amino acid residues in aspartate aminotransferase. The peptides are listed in Table I in the order in which they occur in the intact molecule. The list contains all the fragments obtained including some cases where the same region of the structure is contained in more than one peptide owing to the low specificity of pepsin. Quantitative amino acid compositions of some of the peptides are presented in Table II. These are generally peptides which were not obtained from the first peptic digest; in cases where analogy with previously isolated fragments was evident, only qualitative analyses were carried out.

Peptide RP–21 was obtained in 20 % yield after gel filtration in Sephadex G–25 and an exchange chromatography on SP–Sephadex; in the latter case it was eluted at a basic pH (fig. 1). Edman degradation and dansylation yielded only the first three residues; it is likely that the remaining hydrophobic part was partially lost during extraction with butyl acetate. Digestion of the peptide with carboxypeptidase C yielded: phe, 3.9; pro, 1.0; leu, 1.0, (arg, not calculable). Treatment with amino peptidase yielded: lys, 1.0; arg, 1.9; phe, 2.3; leu, 1.0. These results are consistent with the sequence shown in Table I.

Peptide RP–34 resulted from cleavage C–terminal to glycine, rather than at the preceding glutamine residue which might be expected to be the

favoured point of hydrolysis. The peptide was obtained in low yield (2%) after several purification stages. Sequence analysis gave unclear results in the central part of the peptide (Table I) and the sequence in this region was established on the basis of the quantitative amino acid analysis and by comparison with known tryptic and thermolytic peptides [3], [4].

TABLE I.

List of peptides obtained by limited peptic digestion of aspartate amino transferase.

—— Residues from dansyl Edman method; → Residues from digestion with aminopeptidase; ← Residues from digestion with carboxypeptidase.

RP	1	Ala–Pro–Pro–Ser–Val–Phe
RP	2	Ala–Glu–Val–Pro–Gln–Ala–Gln–Pro–Val–Leu
RP	3	Gly–Val–Gly–Ala
RP	4	Tyr–Arg–Thr–Asx–Asx–Cys–Glx–Pro–Trp–Val–Leu
RP	5	Pro–Val–Val–Arg–Lys–Val–Glu–Gln–Arg–Ile–Ala–Asn–Asn–Ser–Ser–Leu
RP	6	Leu–Pro–Ile–Leu–Gly–Leu
RP	7	Ala–Glu–Phe
RP	8	Ala–Leu–Gly–Asp–Asp–Ser–Pro–Ala–Leu–Glx–Glx
RP	9	Leu–Glx–Glx
RP	10	Arg–Ile–Gly–Ala–Glu–Phe
RP	11	Trp–Tyr–Asn–Gly–Thr–Asn–Asn–Lys–Asp–Thr–Pro–Val–Tyr
RP	12	Val–Ser–Ser–Pro–Thr–Trp–Glx–Asx–His–Asx–Gly–Val–Phe
RP	13	Thr–Thr–Ala–Gly–Phe–Lys–Asp–Ile–Arg–Ser
RP	14	Lys–Asp–Ile–Arg–Ser
RP	15	Tyr–Arg–Tyr–Trp–Asx–Thr–Glx–Lys–Arg–Gly–Leu
RP	16	Phe–Leu–Ser–Asp–Leu
RP	17	Leu–Glx–Asx–Ala–Pro–Glx–Phe
RP	18	Glx–Asx–Ala–Pro–Glx–Phe
RP	19	Val–Leu–His–Ala–Cys–Ala–His–Asx–Pro–Thr–Gly–Thr–Asx–Pro–Thr–Pro–Glx–Glx

Continued: TABLE I.

RP	20	Ser–Val–Met
RP	21	Lys–Arg–Arg–Phe–Leu–Phe–Pro–Phe–Phe
RP	22	Asp–Ser–Ala–Tyr–Gln–Gly–Phe–Ala–Ser–Gly–Asn–Leu
RP	23	Asp–Ser–Ala
RP	24	Tyr–Gln–Gly–Phe–Ala–Ser–Gly–Asn–Leu
RP	25	Ala–Ser–Gly–Asn–Leu
RP	26	Gly–Asn–Leu
RP	27	Ala–Ile–Arg–Tyr
RP	28	Phe–Val–Ser–Glu–Gly–Phe
RP	29	Val–Ser–Glu–Gly–Phe
RP	30	Cys–Ala–Gln–Ser–Phe–Ser–Lys–Asn
RP	31	Ser–Lys–Asn
RP	32	Tyr–Asx–Glx–Arg–Val–Gly–Asn–Leu
RP	33	Thr–Val–Val–Ala–Lys–Glu–Pro–Asp–Ser–Ile–Leu
RP	34	Gln–Lys–Ile–Val–Arg–Val–Thr–Trp–Ser–Asx–Pro–Pro–Ala–Gln–Gly
RP	35	Asp–Arg–Ile–Leu–Ser
RP	36	Arg–Ala–Arg–Leu
RP	37	Glu–Ala–Leu–Lys–Thr–Pro–Gly–Thr–Trp–Asx–His–Ile–Thr–Asp–Gln
RP	38	Leu–Lys–Thr–Pro–Gly–Thr–Trp–Asx–His–Ile–Thr–Asp–Gln
RP	39	Ile–Gly–Met
RP	40	Ile–Asx–Glx–Lys–His–Ile–Tyr–Leu
RP	41	Ala–Val–Thr–Lys–Ile–Gln
RP	42	Lys–Ile–Gln

Similarly, complete sequence data was not obtained for peptides RP–4, RP–6, RP–11 and RP–12, and the structures shown were derived partly by comparison with previously established sequences. The structures are in all cases consistent with the quantitative amino acid analyses.

TABLE II.

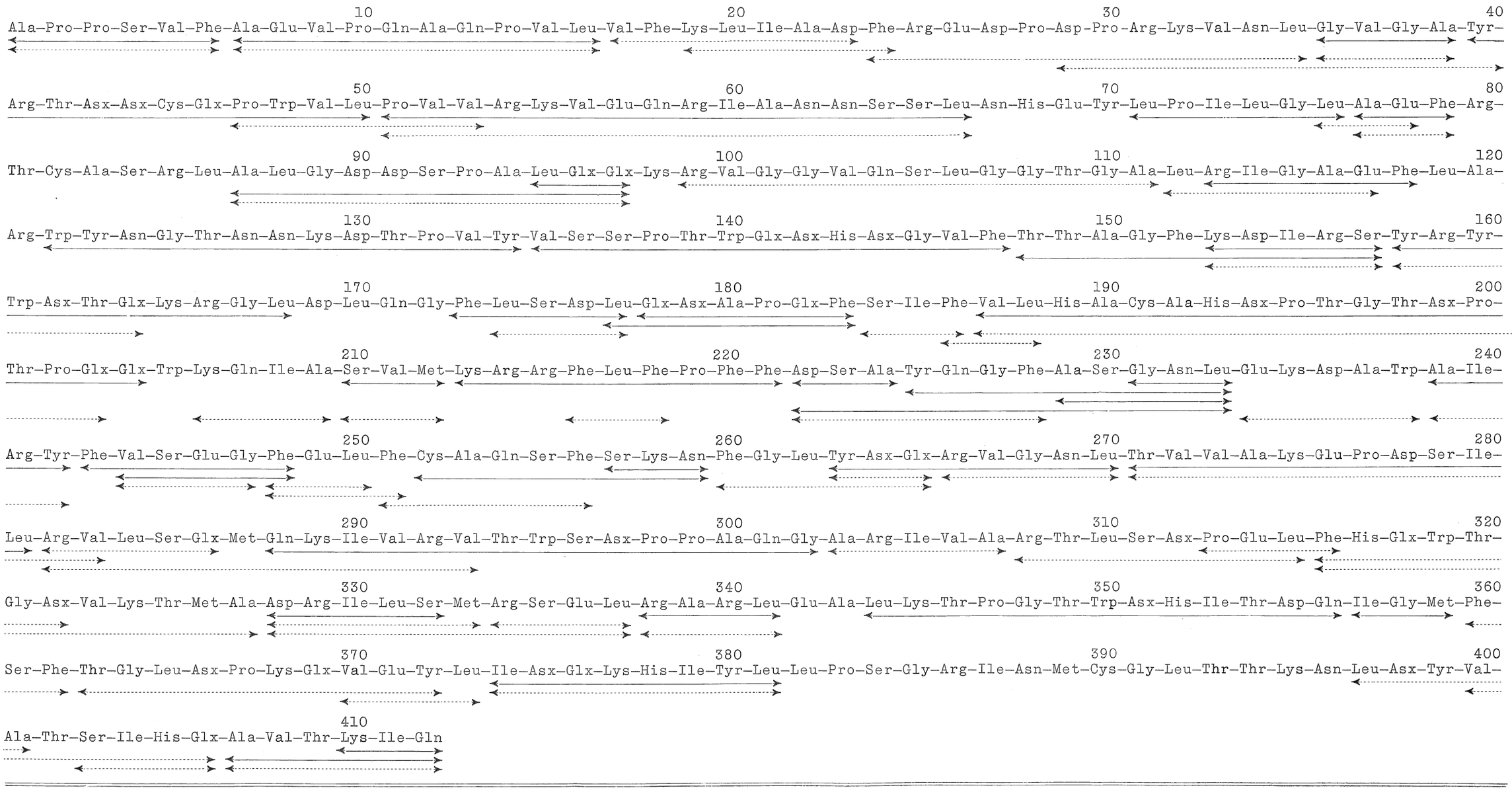
Amino acid composition of peptides.

S = Sephadex G–25; SP = SP Sephadex; Ch = chromatography; E = electrophoresis.

	RP 2	RP 3	RP 4	RP 6	RP 9	RP 10	RP 11	RP 12	RP 13	RP 15	RP 16	RP 17	RP 19	RP 21	RP 22	RP 24	RP 25	RP 30	RP 32	RP 33	RP 34	RP 35	RP 36	RP 37	RP 38	RP 39	RP 41	RP 42
His	—	—	—	—	—	—	—	1.1	—	—	—	—	1.9	—	—	—	—	—	—	—	—	—	—	0.9	1.0	—	—	—
Lys	—	—	—	—	—	—	1.0	—	0.9	0.9	—	—	—	0.8	—	—	—	1.0	—	0.9	0.8	—	—	1.2	1.2	—	1.0	0.8
Arg	—	—	1.0	—	—	0.9	—	—	0.9	2.1	—	—	—	2.0	—	—	—	—	0.8	—	1.0	1.1	2.0	—	—	—	—	—
CMCys	—	—	1.0	—	—	—	—	—	—	—	—	—	1.1	—	—	—	—	0.8	—	—	—	—	—	—	—	—	—	—
Asp	—	—	2.3	—	—	—	4.1	2.1	1.0	1.0	1.2	1.1	2.0	—	2.1	0.8	1.1	1.0	1.7	1.0	1.1	1.1	—	1.8	2.2	—	—	—
Thr	—	—	1.2	—	—	—	1.9	0.9	1.9	0.8	—	—	2.9	—	—	—	—	—	—	1.0	1.0	—	—	2.9	2.9	—	0.8	—
Ser	—	—	—	—	—	—	—	2.0	1.0	—	1.2	—	—	—	2.2	0.8	0.9	1.8	—	1.0	1.0	0.9	—	—	—	—	—	—
Glu	2.9	—	1.0	—	2.2	1.1	—	1.1	—	1.1	—	2.1	2.1	—	1.2	1.1	—	1.2	1.0	1.1	2.0	—	—	2.0	1.2	—	1.2	1.0
Pro	1.7	—	1.1	1.1	—	—	0.9	0.9	—	—	—	1.0	2.7	0.8	—	—	—	—	—	1.0	1.8	—	—	0.9	0.9	—	—	—
Gly	—	2.0	—	1.2	—	1.0	1.1	1.0	1.1	1.1	—	—	1.3	—	2.1	2.0	1.1	—	1.1	—	1.2	—	—	1.1	1.2	1.1	—	—
Ala	2.0	1.0	—	—	—	1.0	—	—	1.0	—	—	1.0	2.0	—	2.0	1.0	1.0	1.0	—	1.0	1.0	—	1.0	1.0	—	—	1.0	—
Val	1.9	1.0	0.9	—	—	—	1.1	2.0	—	—	—	—	1.2	—	—	—	—	—	1.0	2.1	1.8	—	—	—	—	—	0.9	—
Met	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.8	—	—
Ile	—	—	—	1.0	—	0.8	—	—	1.0	—	—	—	—	—	—	—	—	—	—	1.0	0.8	1.0	—	1.0	0.9	1.0	1.0	0.9
Leu	1.0	—	1.0	3.0	1.0	—	—	—	—	1.0	2.0	0.9	1.0	1.0	1.0	1.0	1.0	—	1.0	1.0	—	1.0	1.1	1.1	1.0	—	—	—
Tyr	—	—	1.0	—	—	—	2.0	—	—	1.8	—	—	—	—	1.0	0.8	—	—	1.2	—	—	—	—	—	—	—	—	—
Phe	—	—	—	—	—	1.1	—	0.8	1.0	—	0.9	0.9	—	4.1	0.9	1.0	—	1.0	—	—	—	—	—	—	—	—	—	—
	S SP	S SP	S Ch E	S SP Ch	S SP Ch E	S SP Ch E	S SP Ch E	S SP Ch E	S SP Ch E	S SP E	S SP Ch	S SP Ch E	S Ch E	S SP	S SP Ch E	S SP Ch E	S SP Ch E	S SP Ch	S SP Ch E	S SP Ch E	S SP Ch E	S SP Ch E	S SP Ch E	S SP Ch	S SP Ch	S Ch E	S Ch E	S SP Ch E

TABLE III.

Positions of peptides obtained in the present work (solid line) and those of peptides obtained from the previous peptic digestion (broken line).



DISCUSSION

The main purpose of the restricted peptic digest was to obtain the necessary overlapping fragments to allow completion of the primary structure of aspartate aminotransferase. Although the initial peptic digest had yielded useful information, it was clear that the digest was too extensive and that some regions of the molecule had been largely degraded. Hence attempts were made to use pepsin in a restricted digest while at the same time carrying out studies with enzymes of different specificities. Preliminary experiments showed that drastically reducing both the time of incubation and the ratio of pepsin to aspartate amino-transferase resulted in less extensive cleavage while retaining the important characteristic of solubility of the digest.

In Table III are shown the positions of the peptides obtained in the present work and, for comparison, those from the previous peptic digest.

Some of the peptides deserve special comment in that they provided essential overlaps of confirmed regions of the structure which are in doubt. Peptide RP 21 (residues 213–221) provided an overlap between a thermolytic peptide with the sequence Val–Met–Lys–Arg–Arg–Phe and a chymotryptic peptide Leu–Phe–Pro–Phe–Phe–Asp–Ser–Ala–Tyr [4]. Peptide RP 21 contains several potential peptic cleavage points and was not surprisingly only isolated from the restricted pepsin digest. This also applies to peptide RP 13 (residues 148–157) which supplied the essential overlap between our previously published composite fragments A and B [4], [1]. The C–terminal residue of RP 13 also served to establish the identity of residue 157 as serine; this point had been in doubt from our previous work. Peptide RP 34 overlaps the composite fragments G and H [4], while peptide RP 37 formed a continuation of composite fragment H at its C–terminus and allowed incorporation into the structure of a previously unplaced thermolytic peptide T1 33 [4]. The overlap between peptides RP–37, RP–39 and composite fragment I came from the use of a new protease with specificity for cleavage at lysine [1].

A comparison of the results of the extended and restricted pepsin digests is of interest (Table III). In a small number of cases shorter fragments were obtained in the restricted digest than in the extended one; this may reflect different degrees of accessibility of these regions of the protein to pepsin during the two digests due to differences in the physical state of the suspension in formic acid. More frequently, fragments were formed which were larger than those in the extended digest; examples are peptides RP 4 (residues 40–50), RP 6 (residues 71–76), RP 13 (residues 148–157), RP 15 (residues 158–168), RP 21 (residues 213–221) and RP 32 (residues 263–270). The great importance of some of these peptides in establishing overlaps has already been emphasized.

Fragments were also isolated which were not obtained in the extended peptic digest, for example peptides RP 11, RP 12, RP 37 and RP 39. Failure to obtain these fragments during earlier work may be attributable to the fact that the regions of the protein from which they arise were largely degraded during the extended digest, or to inability to extract the fragments with the

particular purification techniques employed. It is of interest in the latter context that ion exchange chromatography on Sephadex was used in the present work; this method appears to give better yields of peptides than does the use of paper chromatography or electrophoresis, particularly for peptides with a high content of aromatic residues.

Even though the time of exposure to pepsin was short some parts of the protein showed multiple fragmentation. For example, peptide RP 16, 17 and 18 all arise from the region of residues 173 to 183. Different yields of the three fragments ranging from 6% to 1 % were obtained and it is tempting to draw conclusions about preferential points of cleavage from these data. However, the fact that the peptides were isolated by different procedures and that handling losses are not known makes comparisons difficult. For example, peptide RP 16 was recovered in 6% yield after purification by gel filtration and paper chromatography whereas purification of RP 17 (1% yield) required four steps. Obviously the lower yield of RP 17 may be attributed to greater handling losses rather than a smaller degree of hydrolysis of the appropriate peptide bonds. The part of the molecule between residues 222 and 234 similarly showed many cleavage points, giving rise to peptides RP 22, 23, 24, 25 and 26. This extensive fragmentation greatly increased the difficulty of isolation of peptides but seems to be an unavoidable complication when using pepsin for structural studies.

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