ADDENDUM

Corrections to the Amino Acid Sequence of Bovine Chymotrypsinogen A

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Reinvestigation of the amino acid sequence of bovine chymotrypsinogen A suggests that the amino acid sequence at the *N*-terminus of the B-chain (residues 16–19) is -Ile-Val-Asn-Gly- rather than -Ile-Val-Gly-Asp- and that Ser-215 should be deleted.

Examination of the homologies of amino acid sequence between the bovine pancreatic 'serine' proteinases (Hartley, Brown, Kauffman & Smillie, 1965) has prompted us to reinvestigate three areas of the sequence of bovine chymotrypsinogen A (Hartley, 1964*a*).

Val-Gly-Ile-Val-Ser-Ser-Trp (*residues* 210–216). The evidence for this sequence depended on two peptides from the tryptic 'core' of the C-chain of reduced carboxymethylated α-chymotrypsin (Hartley, 1964*a*,*b*). Peptide 1 (Table 1) was a chymotryptic peptide that gave free tryptophan when incubated with carboxypeptidase A. The sequential 'dansyl'–Edman technique (Gray & Hartley, 1963) gave DNS-Val* (++) plus DNS-Val-Gly (+) with the original peptide, DNS-Gly (++) plus DNS-Val (trace) after one step of

* Abbreviations: DNS, 1-dimethylaminonaphthalene-5-sulphonyl; in amino acid sequences Asn and Gln refer to asparagine and glutamine residues respectively.

Table 1. *Val-Gly-Ile-Val-Ser-Trp* (*residues* 210–215)

The amino acid analyses are uncorrected for destruction or incomplete hydrolysis. Ratios are expressed relative to the residues underlined.

Method	Peptide						Reference
	1. Val	Gly	Ile	Val	Ser, (Ser?)	Trp	
Sequential 'dansyl'–Edman	... →	→	→	→			Hartley (1964*a*)
18hr. acid hydrolysate							
μmole	0·021	0·024	0·011	0·010	0·033	+	
Ratio	0·9	1·0	0·5	0·4	1·4		
	2. Ile	Val	Ser,	(Ser?)	Trp		
Sequential 'dansyl'–Edman	... →	→	→	-->			Hartley (1964*b*)
Carboxypeptidase A	... ...				←		
36hr. acid hydrolysate							
μmole	0·050	0·057	0·088				
Ratio	0·9	1·0	1·6		+		
12hr. acid hydrolysate							This work
μmole	0·046	0·048	0·112				
Ratio	0·4	0·4	1·0		+		
144hr. acid hydrolysate							
μmole	0·106	0·114	0·061				
Ratio	1·0	1·0	0·5		+		
Leucine aminopeptidase							
μmole	0·083	0·088	0·085				
Ratio	1·0	1·0	1·0		+		

the Edman procedure, DNS-Ile-Val (++) after the second Edman step, and DNS-Val (++) after the third step (Table 1); (++), (+) and (trace) throughout this Addendum indicate qualitatively the relative fluorescent intensities on paper. Amino acid analysis of an 18hr. acid hydrolysate was taken to indicate a content of two serine residues, but is equivocal because both Val-Gly and Ile-Val are resistant to cleavage by acid, whereas serine is destroyed during acid hydrolysis.

Sequential 'dansyl'–Edman analysis of the chymotryptic peptide 2 gave DNS-Ile-Val (++) with the original peptide, DNS-Val (++) after one round of Edman cleavage, and DNS-Ser (++) plus DNS-Val (trace) after two rounds. After the third round only a trace of DNS-Ser was observed. Amino acid analysis of a 36hr. acid hydrolysate was taken to indicate a composition (Ile,Val,Ser_2,Trp); but, in retrospect, this is equivocal.

Walsh, Kauffman, Kumar & Neurath (1964) found that the homologous sequence in trypsin is Ile-Val-Ser-Trp. Therefore, although the sequence Ile-Val-Ser-Ser-Trp given by Hartley (1964*a*) is apparently confirmed by two other Laboratories (Keil & Šorm, 1964; M. Rovery, personal communication), we decided to reinvestigate the composition of peptide 2 (Table 1). Identical samples were hydrolysed for 12hr. and 114hr. with 5·7N-hydrochloric acid at 108° *in vacuo* or incubated for 5hr. at 37° in 0·02M-*N*-ethylmorpholine–acetate buffer, pH 8·0, containing manganese dichloride (0·1mM), with 0·1mg. of leucine aminopeptidase prepared by the method of Spackman, Smith & Brown (1955). A parallel leucine aminopeptidase blank gave no detectable amino acids when analysed. The results (Table 1) indicate a composition (Ile,Val,Ser,Trp). The most reliable estimate from the acid hydrolysates is to compare the yield of serine after 12hr. (0·112μmole) with the yield of valine (0·114μmole) after 114hr. hydrolysis. Leucine aminopeptidase gave equimolar isoleucine, valine and serine, and paper electrophoresis showed free tryptophan with no detectable sign of a Ser-Trp dipeptide. Hence we should delete the second serine in position 215 of the sequence given by Hartley (1964*a*).

N-Terminus of the B-chain (residues 16–19). This section of the sequence is very important because it represents the new *N*-terminus released by the tryptic activation of chymotrypsinogen A. Rovery, Poilroux, Yoshida & Desnuelle (1957) isolated DNP-Ile-Val-Gly from a partial hydrolysate of DNP-δ-chymotrypsin. The evidence of Hartley (1964*a*) tended to confirm this sequence, since sequential 'dansyl'–Edman analysis of a chymotryptic peptide derived from the *N*-terminus of the carboxymethylated B-chain of α-chymotrypsin had given DNS-Ile-Val (++) with the whole peptide and DNS-Val (++) after one round of Edman cleavage. At the second round, however, only traces of DNS-Gly and DNS-Asp were obtained, but a further Edman step liberated DNS-Asp (+) and traces only of DNS-Gly. Experience in this Laboratory has shown that poor yields of DNS-Gly are frequently found after Edman cleavage, perhaps because cyclization is incomplete. The appearance of DNS-Asp in the fourth position therefore suggested the sequence Ile-Val-Gly-Asp-. An alternative explanation is possible: in a sequence Ile-Val-Asn-Gly-, cyclic-imide formation from asparagine (Naughton, Sanger, Hartley & Shaw, 1960) during acid treatment at the second Edman step could block cyclization to the asparagine phenylthiohydantoin. Subsequent hydrolysis of this cyclic imide would yield *N*-terminal aspartic acid during further stages of the degradation. A poor yield of DNS-Gly in the fourth 'dansyl' step could therefore give the observed result: DNS-Asp (+) plus DNS-Gly (trace). This reinterpretation was stimulated by the observation by Furka, Smillie, Stevenson & Parkes (1966) that residues 16–19 in bovine chymotrypsinogen B are -Ile-Val-Asn-Gly-. We therefore sought to redetermine the *N*-terminal sequence of the B-chain of chymotrypsin A, by treating α-chymotrypsin with fluorodinitrobenzene and isolating the DNP-peptide derived from residues 16–19.

α-Chymotrypsin (25mg.) was dissolved in 8ml. of 8·5M-urea at pH 2·5. The solution was then treated at 37° and pH 8·0 with 0·1ml. of fluorodinitrobenzene in 0·5ml. of ethanol in a pH-stat: alkali uptake was complete in 2hr. The excess of fluorodinitrobenzene was extracted with ether and the solution adjusted to pH 3 and dialysed against 0·01N-hydrochloric acid at 2° overnight. A yellow precipitate formed, which was spun down and washed with acetone and ether.

The precipitate of DNP-protein was dissolved in 0·2ml. of 98% (v/v) formic acid and added to 4ml. of water containing 2·5mg. of pepsin. Digestion took place overnight at 37°. The digest was extracted three times with ethyl acetate until no more yellow colour was removed, and the ethyl acetate evaporated to dryness *in vacuo*.

A test high-voltage paper electrophoresis showed that these DNP-(peptic peptides) were rather heterogeneous. They were therefore redigested with papain (0·1mg.) in 0·2ml. of 0·1M-pyridine–acetate buffer, pH 6·5, containing mercaptoethanol (0·1M), overnight at room temperature. The DNP-peptides were extracted from the digest at pH 3 with ethyl acetate and evaporated to dryness. They were then submitted to high-voltage paper electrophoresis as a 5cm. band on Whatman 3MM paper in 0·1M-pyridine–acetate buffer, pH 6·5, in a cooled flat-plate apparatus. The only anionic yellow bands

were dinitrophenol (mobility 0·80 relative to aspartic acid marker), a major peptide band of DNP-(peptide 1) (mobility 0·47) and a trace band of DNP-(peptide 2) (mobility 0·53). Samples of DNP-(peptide 1) and DNP-(peptide 2) were hydrolysed with 5·7N-hydrochloric acid at 108° for 20hr. The DNP-amino acids were extracted from the acid hydrolysates with ether and chromatographed two-dimensionally on paper in 2-methylbutan-2-ol–2N-ammonia (4:1, v/v) followed by 1·5M-sodium phosphate buffer, pH 6·0. DNP-(peptide 1) gave a mixture of DNP-Ile and DNP-Ile-Val, whereas DNP-(peptide 2) gave DNP-Ala. The aqueous phases were analysed by ion-exchange chromatography (Spackman, 1963): DNP-(peptide 1) gave aspartic acid (0·042μmole) and valine (0·026μmole) (the latter is probably low because of incomplete hydrolysis of DNP-Ile-Val). Negligible free amino acids were found in DNP-(peptide 2). Treatment of DNP-(peptide 1) with carboxypeptidase A followed by high-voltage paper electrophoresis gave asparagine and traces of valine. Hence DNP-(peptide 1) appears to be DNP-Ile-Val-Asn and DNP-(peptide 2) is probably free DNP-Ala from the *N*-terminus of the C-chain of α-chymotrypsin.

Hence we must write the sequence of residues 16–19 as Ile-Val-Asn-Gly-.

Thr-Gly-Phe-His-Phe-Cys (*residues* 37–41). The evidence for the sequence of bovine chymotrypsinogen A (Hartley, 1964*a*) is rather weak in this region, since no positive 'overlap' of the peptide Thr-Gly-Phe and His-Phe is described. However, Keil & Šorm (1964) describe a peptide corresponding to residues 37–41 above, derived from a tryptic digest of the aminoethylated B-chain of α-chymotrypsin. Because of the importance of the His-40 sequence in the mechanism of action of chymotrypsin, we undertook the isolation of this 'overlap' peptide.

Chymotrypsinogen A (250mg.) in 5ml. of N-acetic acid was treated with 5g. of urea, diluted to 10ml. and adjusted to pH 8·7 with solid tris. The solution was allowed to react overnight at room temperature under nitrogen in a Thunberg tube with 0·1ml. of mercaptoethanol. Ethyleneimine (0·6ml.) and acetic acid (0·4ml.) were added and the mixture allowed to react until no free thiol was detectable with sodium nitroprusside (about 2hr. at room temperature). The precipitate that appeared after dialysis was washed with water and digested overnight with 1·5mg. of trypsin in 7ml. of 0·1% ammonium hydrogen carbonate (the trypsin had been treated for 5hr. at 2° and pH 8·0 with 0·1mole of diphenylcarbamoyl chloride/mole of trypsin to inhibit traces of contaminating chymotrypsin; Erlanger & Cohen, 1963). The precipitate remaining in the digest was spun off.

High-voltage paper electrophoresis of a sample of the digest at pH 6·5 showed only two bands of peptide that gave a characteristic histidine colour with Pauly reagent (Dent, 1947). Their mobilities relative to lysine were 0·16 and 0·43. The most cationic of these was further purified by paper electrophoresis at pH 3·5 and at pH 6·9. Amino acid analysis showed that its composition was (Thr, Gly, Phe, His, Phe, aminoethylcysteine), confirming the composition described by Keil & Šorm (1964). Hence the sequence -Thr-Gly-Phe-His-Phe-CyS- proposed by Hartley (1964*a*) for residues 37–41 of bovine chymotrypsinogen A appears to be correct.

There are typographical errors in a previous report of the chymotrypsin A and trypsin sequences (Hartley *et al.* 1965). In Table 1 of that paper, residues 110–111 of chymotrypsinogen A were quoted as -Thr-Ser- instead of -Ser-Thr- and residues 135–136 of trypsinogen as -Gln-Thr instead of -Thr-Gln-. Also, Glu should have been written instead of Glx at positions 78 and 81 of trypsinogen, and Asp instead of Asx at position 80.

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