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On the purity of 3X-recrystallised bovine α-chymotrypsin

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The results of an electrospray-mass spectrometric analytical study of aqueous solutions of fifteen commercial samples of 3X-recrystallised bovine α-chymotrypsin are presented and discussed. It was found that only six samples were predominantly α-chymotrypsin and that two samples contained no α-chymotrypsin at all. The remaining seven samples were found to be mixtures of α-chymotrypsin with other chymotrypsins and, in some cases, neochymotrypsinogens. The majority of the results are rationalised in terms of previously postulated and/or observed products of proteolytic activation of bovine chymotrypsinogen A. However, evidence is also presented for the presence in many of the samples of three new serine proteases, of significantly lower molecular masses than α-chymotrypsin, which cannot at present be explained. The paper is concluded with a brief discussion of the implications of the analytical findings for enzymological studies.

In a recently published study (1) we employed the new analytical technique, electrospray-mass spectrometry (ES-MS) to confirm the existence of certain relatively stable covalent 0-acyl enzyme intermediates for a number of serine proteases. During the course of this study, we found that the sample of commercial 3X-recrystallised bovine α-chymotrypsin used was not pure in that it consisted of two enzymes, with α-chymotrypsin forming the minor component and another (unidentified) serine protease of molecular mass M=25,450 daltons as the major component. Whilst clearly it would be interesting to determine the identify of the latter, this experimental finding raised an even more important general question as to the purity of this grade of enzyme which is widely used as the starting material for most enzymological studies. Therefore the primary aim of the present study was to determine the purity of various samples of commercial 3X-recrystallised bovine α-chymotrypsin.

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THEORY

Since bovine α-chymotrypsin is prepared using enzymatic procedures from chymotrypsinogen A obtained from bovine pancreas, we first carefully studied the literature on this subject in order to ascertain the possible utility of the ES-MS technique. As we have shown elsewhere, the primary attribute of this technique is its ability to rapidly yield accurate (0.005%) molecular mass data for solutions of pure proteins (2) and for protein mixtures (3) without having to employ chromatographic purification procedures, provided that the molecular masses of the components with Mr ≈25kDa, differ by some 12-14 daltons. Accordingly we calculated the exact molecular masses of all of the known and postulated enzymatic products of bovine chymotrypsinogen A as reported in the excellent review of this subject by Bender and Killheffer (4) and our results are given in Table 1. A study of column 3 (status of labile bonds in Table 1) shows that a total of seven enzymatically inactive neochymotrypsinogens and seven enzymatically active chymotrypsins are theoretically possible each of which has been assigned a unique notation (Column 2). In terms of accurate molecular

Table 1. Listing of trivial names, suggested notation, status of labile bonds, molecular formula and molecular masses of neochymotrypsinogens and chymotrypsins formed from bovine chymotrypsinogen A

Trivial Name	Notation	Status of labile bonds*				Molecular Formula					Molecular Mass (daltons)
		R-I 15-16	L-S 13-14	Y-T 146-147	N-A 148-149	C	H	N	O	S	
Chymotrypsinogen	A	0	0	0	0	1127	1773	307	353	12	25,656.1
Neochymotrypsinogen	A_1	0	0	0	1	1127	1775	307	354	12	25,674.1
threo-	A_2	0	0	1	0	1127	1775	307	354	12	25,674.1
ala-	A_3	0	0	1	1	1119	1762	304	350	12	25,458.9
None	A_4	0	1	0	0	1127	1775	307	354	12	25,674.1
None	A_5	0	1	0	1	1127	1777	307	355	12	25,692.1
None	A_6	0	1	1	0	1127	1777	307	355	12	25,692.1
ala, ser-	A_7	0	1	1	1	1119	1764	304	351	12	25,476.9
π-chymotrypsin	A_0	1	0	0	0	1127	1775	307	354	12	25,674.1
None	A_1	1	0	0	1	1127	1777	307	355	12	25,692.1
None	A_2	1	0	1	0	1127	1777	307	355	12	25,692.1
None	A_3	1	0	1	1	1119	1764	304	351	12	25,476.9
δ-	A_4	1	1	0	0	1119	1758	302	351	12	25,430.9
None	A_5	1	1	0	1	1118	1760	302	352	12	25,448.9
α_1, or χ-	A_6	1	1	1	0	1118	1760	302	352	12	25,448.9
α- and γ-	A_7	1	1	1	1	1110	1747	299	348	12	25,233.7

*Key: 1, denotes broken bond; 0, denotes intact bond.

masses, see Column 12, however,.only four distinct masses are obtained for the neochymotrypsinogens since three of them (A_1-; A_2-; A_4-) have the same Mr = 25,674.1 daltons but six distinct masses are obtained for the chymotrypsins because only two (A_1-; A_2-) possess the same Mr = 25,692.1 daltons. Further consideration of the Mr column of Table 1 shows that, because of their fortuitous identity, a total of only seven distinct Mr's are available to distinguish between the total of fourteen proteolytic products of bovine α-chymotrypsinogen. However, if the ES-MS technique is applied first to a solution of an α-chymotrypsin, and then if heterogeneity is detected, to the same solution which had been reacted with an appropriate aliquot of a solution of cinnamoyl imidazole (CI) the identification problem is improved. Thus, in the latter experiment the masses of any active chymotrypsin species in the solution would be expected to increase by 130.1 daltons, corresponding to the mass increase resulting from the formation of the 0-cinnamoyl chymotrypsin, whereas the masses of any inactive chymotrypsinogen(s) would remain constant. By this expedient of performing two consecutive sets of ES-MS experiments a total of ten of the fourteen possible products of proteolytic cleavage of bovine chymotrypsinogen would be measurable and identifiable if they were present in a solution of an impure α-chymotrypsin.
The essential validity of these theoretical predictions will now be demonstrated by consideration of the results of an ES-MS analysis of various distinct samples of commercial 3X-recrystallised α-chymotrypsin.

EXPERIMENTAL AND RESULTS

A collection of 15 samples of 3X-recrystallised bovine α-chymotrypsin was obtained from a variety of commercial and academic sources, full details of which are listed in column 1 of Table 2. The ES-MS of solutions of each of the samples in acetonitrile/water - 1% formic acid solutions (50/50 v/v) were first measured on a VG Bio Q triple quadrupole mass spectrometer equipped with an electrospray inlet system and data handling system which was calibrated using horse heart myoglobin Mr = 16,951.48 Da (1) and the transformed computed Mr's are summarised in Table 2. When sample heterogeneity was found the enzyme sample was reacted with a solution of cinnamoyl imidazole (Sigma) as detailed previously (1) and the reaction product mixture was again analysed by ES-MS. In this latter case the mass of any reactive enzyme species was calculated by subtracting 130.1Da from the measured mass and the results of these experiments are indicated in column 2 of Table 2 by the addition of (CI) after the study sample number. A study of Table 2 shows that it was possible to assign all of the measured masses to molecular species which have been labelled in the notation given in column 2 of Table 1.

Table 2. Measured molecular masses and relative ion abundance (%) of assigned species present in aqueous acetonitrile-formic acid solutions of samples of 3x-recrystallised α-chymotrypsin obtained from a number of commercial sources, together with the calculated masses of the assigned molecular species

Sample source Lot/Batch Number	Study Sample Number	Names and molecular masses (daltons) of assigned species						
		Active Species				Inactive Species		
		A_7-CT(α) 25,233.7	A_4-CT(δ) 25,430.9	A_5-CT A_6CT(a_1) 25,448.9	A_2-CT A_1-CT 25,692.1	A_3-CTogen 25,458.9	A_7-CTogen 25,476.9	A_1-, A_2-, A_4-CTogen 25,674.1
S128F-8035	1	25,231.8(15%)	-	25,450.5(100%)	-	-	-	25,674.9(48%)
S105C-8065	2	25,232.6(100%)	-	-	-	-	-	-
S15F-8160	3	25,235.0(5%)	25,431.1(100%)	25,448.1(80%)	25,690.0(30%)	-	-	25,672.9(65%)
	3+(Cl)	25,234.9(5%)	25,430.2(60%)	25,448.4(100%)	25,693.7(30%)	-	-	25,675.1(85%)
S36F-8115	4	25,235.1(100%)	25,433.3(30%)	25,451.6(40%)	-	-	-	-
	4+(Cl)	25,234.0(100%)	25,429.9(30%)	25,446.7(40%)	-	-	-	-
S54F-8020	5	Absent	25,430.5(80%)	25,451.8(95%)	25,690.0(50%)	-	-	25,676.1(100%)
	5+(Cl)	Absent	25,432.0(70%)	25,451.5(100%)	25,689.8(40%)	-	-	25,675.3(80%)
S82F-8010	6	25,232.4(100%)	-	-	-	-	25,476.8(20%)	-
	6+(Cl)	25,234.2(100%)	-	-	-	-	25,476.5(35%)	-
S71H-7110	7	25,235.6(100%)	25,433.1(20%)	25,448.8(25%)	-	-	-	-
	7+(Cl)	25,235.2(100%)	25,429.7(20%)	25,448.2(25%)	-	-	-	-
S(None)A.P.	8	25,234.0(95%)	-	25,448.6(100%)	-	-	-	-
	8+(Cl)	25,236.3(95%)	-	25,451.0(100%)	-	-	-	-
N.27	9	25,233.5(100%)	-	-	-	-	-	-
N.29	10	25,235.5(100%)	-	-	-	-	-	-
S4129	11	25,233.3(100%)	-	-	-	25,455.3(20%)	25,475.0(20%)	-
	11+(Cl)	25,234.5(100%)	-	-	-	25,451.4(30%)	25,479.8(30%)	-
B12766922-54	12	Absent	25,433.0(100%)	-	-	-	-	-
C.608035	13	25,235.6(100%)	-	-	-	-	-	-
W57K1124	14	25,233.0(100%)	-	-	-	-	-	-
U72808	15	25,233.9(100%)	-	-	-	-	-	-

KEY: CT = Chymotrypsin: CTogen = Chymotrypsinogen: CI = Cinnamoyl Imidazole: S = Sigma: N = Novo: B = Boehringer: C = Calbiochem: AP = Affinity column [Soybean trypsin inhibitor] purified: W = Worthington: U = USA Biochemical Corporation.

DISCUSSION

Three major conclusions may be drawn from the ES-MS experimental results summarised in Table 2. The first is that only six of the fifteen 3X-recrystallised α-chymotrypsin samples, namely Nos. 2, 9, 10, 13, 14, 15 were predominantly α-chymotrypsin (A_7). All of these samples also contained variable amounts of sodium, potassium or sulphate molecular adducts (*i.e.* Mr+23; Mr+39; Mr+98) and the purest of the six, in terms of the lowest percentage of these adducts were samples Nos. 14 and 15 which appeared, by ES-MS, to be identical [Fig. 1].

Secondly, two of the samples (Nos. 5 and 12) contained no α-chymotrypsin (A_7) at all and sample No. 3 contained only a trace of this enzyme. Sample No. 12 was found to be δ-chymotrypsin or A_4-chymotrypsin and a simple explanation of our findings is that the suppliers (Messrs. Boehringer) had mislabelled the sample since it was catalogued and sold as α-chymotrypsin (A4). No such explanation is possible for the other two samples (Nos. 3 and 5) since according to the ES-MS analysis, they were mixtures of A_4-chymotrypsin (δ); A_5- and/or A_6-chymotrypsin; A_1- and/or A_2-chymotrypsin and A_1-/A_2-/A_4-chymotryp-

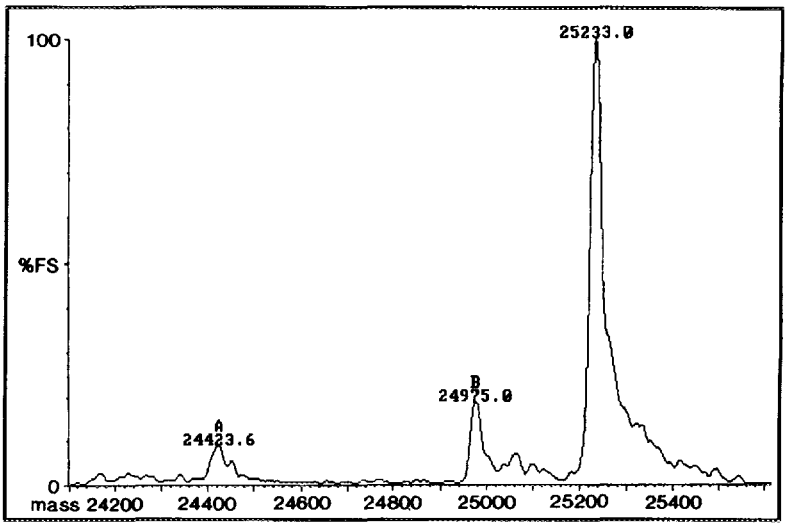

Figure 1. Transformed ES-MS spectrum of 3X-recrystallised-α-chymotrypsin. (Sample 14, Worthington).

sinogen. It should be noted here that the enzymatically active species of Mr = 24,450 Da found in our previous study (1) and discussed in the Introduction on measurement has an Mr = 25,448.91 Da which we have now assigned to A_5- and/or A_6-chymotrypsin. The latter enzyme has been reported previously (5) and has the trivial name α_1-chymotrypsin. In order to support this assignment we also prepared it from A_4-chymotrypsin(δ) and confirmed (by ES-MS) that it reacted with cinnamoyl imidazole.

Thirdly, all of the remaining samples were heterogeneous mixtures of either A_7-chymotrypsin(α) with other chymotrypsins and with or without additional chymotrypsinogens. Thus sample No. 8, which had been subjected to affinity chromatography, contained only A_7-chymotrypsin(α) plus A_5- and/or A_6-chymotrypsin (α_1) and it is possible that this chromatographic treatment had removed any chymotrypsinogens present in the original sample. Samples Nos 4 and 7 appeared to be identical in terms of molecular composition since they both consisted of a mixture of three enzymatic species namely, A_7-; A_4-; and A_5- and/or A_6-chymotrypsin albeit in somewhat different relative proportions. Similarly, samples Nos. 6 and 11 were similar in molecular composition except that sample 11 contained the additional A_3-CTogen species. The final sample whose digitised data is to be discussed, No. 1 contained a small amount of A_7-chymotrypsin(α) and A_5- and/or A_6-chymotrypsin(α_1) plus A_1-/A_2-/A_4-chymotrypsinogen.

In addition to these conclusions drawn from the tabulated ES-MS data, consideration will now be given to some graphical ES-MS data. A study of the transformed ES-MS data (figure 1) shows that two molecular species labelled A and B, of low relative abundance and with M_r's of 24,423.6 and 24,975.0 daltons respectively, were present in the α-chymotrypsin sample No. 14. A careful examination of all of the other transformed ES-MS graphical data revealed that these two molecular species were also present in eight other samples and calculation of the mean M_r's led to the corresponding average values of 24,419.6 and 24,977.6 daltons respectively, for A and B. This finding was also supported by the ES-MS analysis of a commercial sample of diisopropylphospho-(DFP)-α chymotrypsin (*ex* Sigma) shown in Figure 2. This figure shows the presence of three molecular masses (A'; B'; C') of low relative abundance with M_r=24,586.6; 25,143.5 and 24,798.9 daltons respectively. The molecular species A' and C' may be assigned to the covalent DFP acyl derivatives of the previously observed proteases A and B (Figure 1) by the coincidences of the M_r's obtained by subtracting 164.1 daltons from M_r of A' and B'. By an identical process of subtraction, the molecular species C' may be assigned to a DFP-covalent acyl derivative of a new protease C with M_r=25,634.8 daltons. A subsequent careful study of the remainder of the transformed graphical ES-MS data showed that this molecular species was present, albeit of low relative abundance, in six of the samples analysed and this led to the calculation of a mean M_r=24,630 daltons for protease C. Further study of the transformed ES-MS graphical data obtained from the acylation experiments proved, from the mass changes, that all of these three molecular species were acylated by cinnamoyl imidazole.

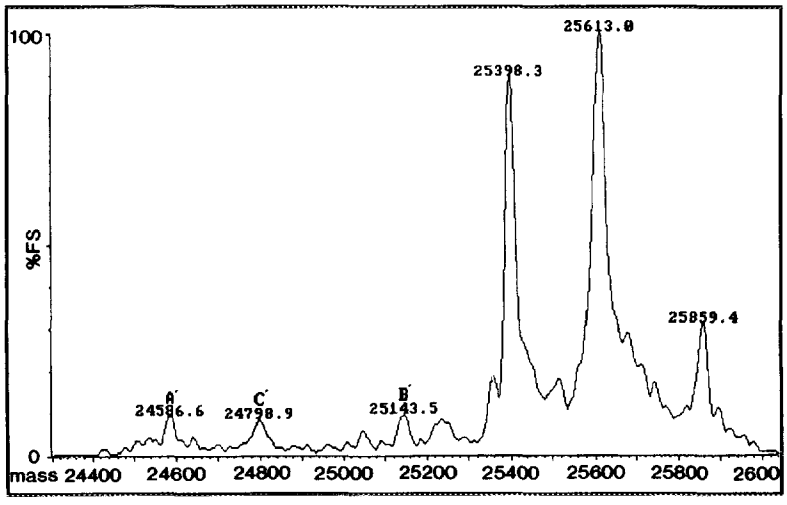

Figure 2. Transformed ES-MS spectrum of DFP-α-chymotrypsin (Sigma).

It may therefore be concluded that three new serine proteases labelled A, B and C were present in a number of the α-chymotrypsin samples analysed. The three corresponding molecular masses were not included in Table 2 because they cannot be explained in terms of the chymotrypsinogen activation scheme of Bender *et al* (4) summarised in Table 1. The exact nature and structure of these three commonly occurring (impurity) enzymes remains to be discovered and must be ascertained before a complete molecular analysis of 3X-recrystallised α-chymotrypsin can be performed. We are of the considered opinion that these differing patterns of molecular heterogeneity reflect the various methods of production employed by the different manufacturers.

COMMENTS

This study has, we believe, shown in accordance with our theoretical predictions that the ES-MS technique, especially when combined with the cinnamoyl imidazole acylation reaction, can readily and quickly (5-30 mins) determine the purity and molecular composition of 3X-recrystallised α-chymotrypsin samples. In view of the heterogeneity observed, and in some cases of erroneous identity of some of the commercial samples studied, it is recommended that the procedures adopted here should wherever possible be employed before and after any kinetic or structural studies on this enzyme are implemented. Further, it is possible that the well known variable proteolytic cleavage properties of α-chymotrypsin are due to the heterogeneity of the enzyme sample employed. Finally, because of this, and because of the need to fully understand the pattern of the analytical findings presented here we plan to perform a detailed re-investigation of the mechanism of activation of bovine chymotrypsinogen A using ES-MS techniques.

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REFERENCES

1. Ashton, D.S., Beddell, C.R., Cooper, D.J., Green, B.N., Oliver, R.W.A. and Welham, K.J. (1991) *FEBS Letts.* 292, 201-204.
2. Ashton, D.S., Beddell, C.R., Green, B.N. and Oliver, R.W.A. (1992) *Biochem.J.* (submitted).
3. Green, B.N. (1992) *Biochem.J.*, 284, 603-608.
4. Bender, M.L. and Kilheffer, J.V. (1973). In CRC Reviews of Biochemistry, pp149-194. CRC Press.
5. Miller, D.D., Horbett, T.A. and Teller, D.C. (1971) *Biochemistry*, 10, 4641-4648.