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PARTIAL IDENTIFICATION OF A CHYMOTRYPSIN FORMED BY INCUBATION OF BOVINE CHYMOTRYPSINOGEN A WITH PAPAIN

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SUMMARY

A chymotrypsin (EC 3.4.4.5) was isolated from solutions of bovine chymotrypsinogen A incubated with papain. This chymotrypsin appears to be identical with α-chymotrypsin, except for the loss of three residues (Ser_{11}, Gly_{12} and Leu_{13}), split off as a consequence of the selective cleavage of the Leu_{10}–Ser_{11} bond.

Bovine chymotrypsinogen A has been shown to be slowly activated by papain[1]. The interest in this process lies in the fact that papain hydrolyzes, not only basic bonds in N-substituted amides and esters[2], but also an variety of other bonds in synthetic substrates[2], large peptides[3] and proteins[4]. Accordingly, an active chymotrypsin (EC 3.4.4.5) resulting from incubation of chymotrypsinogen with papain has been purified and tentatively identified in the hope that it would be more degraded than α-chymotrypsin.

The incubations of chymotrypsinogen (Worthington, 5 × crystallized) with papain (Worthington, 2 × crystallized) were essentially performed as described in ref. 1. In a typical experiment, 60 mg of chymotrypsinogen in 6.0 ml of 0.1 M acetate buffer (pH 5.0), containing 10 mM EDTA and cysteine were incubated at 23° for a total time period of 96 h with 3 mg of papain added in three portions at times zero, 24 and 48 h. The specific chymotryptic activity, measured by titrimetry against acetyl-L-tyrosine ethyl ester, slowly increased and reached after 72 h a maximal value approximately equal to 25% of that of crystalline α-chymotrypsin. Aliquots were treated with ^{32}P-labeled diisopropylfluorophosphate (DFP) in order to label the active molecules produced after various time periods. The resultant solutions were subjected to equilibrium chromatography on CM-cellulose (Whatman CM-32) at pH 8.5 (M. ROVERY AND J. BIANCHETTA, unpublished experiments). Figs. 1a–1e show that one major and two minor radioactive peaks were obtained under these conditions. The relative proportion of the major component was maximal after 72 h. Large amounts of inert material were visible in all cases.

In subsequent experiments, the 72-h digests were first purified by affinity chromatography[5] using columns of Sepharose 4B coupled with ε-aminocaproyl-D-

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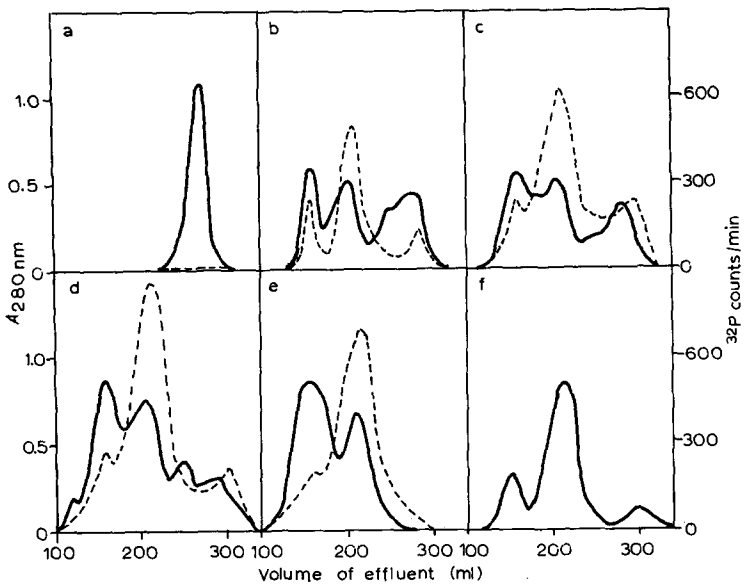

Fig. 1. Equilibrium chromatography of the papain-activated solutions. The 2.0 cm × 40 cm Whatman CM-32 column was equilibrated and eluted by a 5 mM Tris–100 mM sodium acetate buffer (pH 8.5). Flow rate, 30 ml/h. Temp., 25°. (a–e) Samples incubated with papain for 0, 24, 48, 72 and 96 h, respectively. (f) Sample activated for 72 h and previously purified by affinity chromatography (see text). ——, absorbance at 280 nm; — — —, radioactivity of the fractions.

tryptophan methyl ester*. Application of the digests to this column at pH 8.0 resulted in the selective retention of the active molecules which were later eluted by 100 mM acetic acid and subjected as above to equilibrium chromatography. Fig. 1f shows that previous removal of the inert contaminants improved the resolution of the three active peaks on CM-cellulose. The fractions under the major peak were pooled and subjected to a second affinity chromatography. After this step, the specific activity (number of chymotrypsin units per mg proteins (measured spectrophotometrically at 280 nm)) of the purified preparation was 280 as compared to 410 observed for the similarly purified chymotrypsin *a*.

Identification of the chymotrypsin thus obtained was attempted in two ways: (a) Determination of the N- and C-terminal residues, respectively, by condensation of the DFP-inhibited enzyme with fluorodinitrobenzene and by application to the derivative of a hydrogen–tritium exchange method[6]. Isoleucine (0.57 mole/mole, uncorrected) and alanine (0.62) were found on the amino side whereas leucine (5740 counts/min per μmole), tyrosine (4800) and asparagine (5800) were found on the carboxyl side. Identical results were obtained with an inhibited sample of crystalline *a*-chymotrypsin. (b) Isolation and analysis of the short chain A liberated by performic acid oxidation of the enzyme molecule[7]. Table I shows that this chain contained only 10 residues instead of 13 in the corresponding chain of *a*-chymotrypsin. The three missing residues were these of the C-terminal sequence of the chain: Ser_{11}, Gly_{12} and Leu_{13}.

* We are indebted to Dr. C. B. Anfinsen for a gift of this derivative.

TABLE I

AMINO ACID COMPOSITION OF THE A CHAIN OF THE PAPAIN-ACTIVATED CHYMOTRYPSIN

Residue	*Calc. number of residues (24-h hydrolysis) per mole of*	
	Papain-activated chymotrypsinogen	*α-Chymotrypsin (for comparison)*
Ala	0.98	0.94
Cys	0.86	1.07
Glx	1.05	1.02
Gly	0.97	2.17
Ile	0.94	0.97
Leu	0.94	1.88
Pro	2.32	1.93
Ser	0.08	0.98
Val	1.86	2.10

It can therefore be concluded that, as expected, papain activates chymotrypsinogen according to the now classical scheme involving cleavage of the Arg_{15}–Ile_{16} bond. When compared to the formation of α-chymotrypsin, the process appears to involve at least one additional non-inactivating cleavage at the level of the Leu_{10}–Ser_{11} bond. Further work is in progress to ascertain whether the cleavage of this leucine bond results from a papain or from a chymotrypsin attack, to confirm that no other bond is being split during the formation of the major chymotrypsin and to identify the minor active components separated on CM-cellulose.

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