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PREPARATION AND PROPERTIES OF THREE-CHAIN NEOCHYMOTRYPSINOGENS

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SUMMARY

Porcine chymotrypsin C is able to hydrolyze in native bovine chymotrypsinogen A bonds 146 and 148 and also bonds 10 and 13 situated a few residues apart from the "activating" bond Arg_{15}–Ile_{16}. Hydrolysis of bond 13 does not activate chymotrypsinogen. But it is compatible with subsequent activation of the molecule and, therefore, gives rise to a neochymotrypsinogen with three open chains.

Residues Tyr_{146} and Arg_{145} can be removed from the above neochymotrypsinogen by the combined action of carboxypeptidases A and B, without impairing the activability of the molecule. This observation does not confirm the postulation according to which residue Arg_{145} plays a role during chymotrypsinogen activation by trypsin.

INTRODUCTION

The limited proteolysis converting bovine chymotrypsinogen A into several chymotrypsins (chymotrypsin π, δ and α) occur in the two following regions of the zymogen molecule:

Leu_{10}–Ser_{11}–Gly_{12}–Leu_{13}–Ser_{14}–Arg_{15}–Ile_{16}	Sequence I
Thr_{144}–Arg_{145}–Tyr_{146}–Thr_{147}–Asn_{148}–Ala_{149}	Sequence II

Trypsin cleaves bond 15* in Sequence I, inducing the appearance of chymotrypsin π, the first active molecule of the chymotrypsin family[1–5]. On the other hand, chymotrypsin cleaves a total of three bonds[3], either by autolysis in chymotrypsin π, or by direct attack of chymotrypsinogen. Spontaneous autolysis of chymotrypsin π at the level of bond 13 in Sequence I leads to the formation of the stable chymotrypsin

Abbreviations: ATEE, *N*-acetyl-L-tyrosine ethyl ester; ATA, *N*-acetyl-L-tyrosine amide; FDNB, fluorodinitrobenzene; DNP-, dinitrophenyl-.

* For the sake of simplicity, the bonds in bovine chymotrypsinogen A are designated by the number of the preceding residue in the single peptide chain of the precursor. For instance, bond 15 is that connecting residue Arg_{15} to Ile_{16}. Moreover, the numbering of residues in the various chymotrypsins is the same as in chymotrypsinogen A taken as the reference protein.

δ and to the loss of the dipeptide Ser_{14}–Arg_{15} (refs. 1–5). In addition, chymotrypsinogen A is known to be slowly converted by chymotrypsin α into inactive forms, but which can still be activated, called neochymotrypsinogen[3], differing from the native form by the splitting of bonds 146 and 148 in Sequence II. These neochymotrypsinogens generated by chymotrypsin α possess two chains (Cys_1–Tyr_{146} and Ala_{149} (or Thr_{147})–Asn_{245}) held together by disulfide bridges. They are activated by trypsin through the cleavage of bond 15 to produce chymotrypsin α with three chains (Chain A: Cys_1–Leu_{13}; Chain B: Ile_{16}–Tyr_{146} and Chain C: Ala_{149}–Asn_{245}).

In this connexion, it is noteworthy that the tyrosyl and asparaginyl bonds 146 and 148 are readily split during the digestion of chymotrypsinogen A with chymotrypsin α, whereas the leucyl bond 13, which is quite accessible to autolysis in chymotrypsin π, is much more resistant to chymotryptic attack in the precucsor. The consequence is that the attainment of a neochymotrypsinogen in which bond 13 is hydrolyzed has so far proved difficult. The final characterization of a molecule of this sort appears to be important, since bond 13 is situated only two residues apart from the "activating" bond 15.

It will be shown in the present report that, in contrast with what is observed with bovine chymotrypsin α, porcine chymotryspin C, already known for its high affinity for leucyl bonds, readily hydrolyses bonds 10 and 13 in native chymotrypsinogen A. As a result, incubation of bovine chymotrypsinogen A with this enzyme leads to the formation in good yield of a neochymotrypsinogen containing three chains (Cys_1–Leu_{10}; Ser_{14}–Tyr_{146}; Ala_{149}–Asn_{245}). The properties of this new compound clearly demonstrate for the first time that the cleavage of bond 13 does not activate chymotrypsinogen and that it also does not hinder the subsequent activation of the molecule by splitting of bond 15. Moreover, the consequences of the loss of some residues, especially residue Arg_{145}, on the activity of chymotrypsin and on the ability of neochymotrypsinogen to be activated have been investigated.

MATERIALS AND METHODS

Enzyme assays

Chymotryptic activity was determined against *N*-acetyl-L-tyrosine ethyl ester (ATEE) or amide (ATA). With the first substrate, the ordinary titrimetric technique[6] was used. Solutions of the amide (0.5–2.0 mM) in a 50 mM phosphate buffer at pH 7.8 were incubated at 37°C with the enzyme (final concentration 4 μM). Aliquots of 1 ml were removed at 1-min intervals during the first 6 min. They were mixed with 1 ml of ninhydrin reagent and the tubes were plunged into a boiling water bath for 15 min. After addition of 5 ml of ethanol–water mixture (1:1, v/v), the color was measured at 570 nm. For both substrates, V was expressed in $\mu equiv \cdot min^{-1} \cdot mg^{-1}$.

Digestion of chymotrypsinogen A by chymotrypsin α or chymotrypsin C

Bovine chymotrypsinogen A and chymotrypsin α (3 times crystallized) were purchased from Worthington and used without further purification. Porcine chymotrypsin C was obtained from autolyzed pancreas according to the method of Gratecos and Desnuelle[7]. The preparations were found to contain traces of carboxypeptidase A and trypsin activity which were especially detrimental due to the high proportion of the enzyme used during the incubation assays. These contaminants, therefore,

were carefully removed before use by dialysis against 1 mM *o*-phenanthroline for 18 h of 1.5-μM solutions of chymotrypsin C in a 5 mM Tris buffer (pH 8.0) containing *o*-phenanthroline (1 mM) and soya bean trypsin inhibitor (10 μM). 1 mM *o*-phenanthroline and 10 μM trypsin inhibitor were also present during the incubations.

The incubations were performed by treating, at 5°C for 48 h, solutions of chymotrypsinogen A (3.8 μmoles/ml) in a Tris (5 mM)–acetate (100 mM) buffer (pH 8.0) with chymotrypsin α (0.38 μmole/ml) or chymotrypsin C (0.57 μmole/ml) in the presence of 1 M $(NH_4)_2SO_4$. This salt is known[3] to enhance the chymotryptic hydrolysis of bond 148 and, therefore, to induce the formation of a majority of neochymotrypsinogen molecules with a N-terminal alanine (residue 149). The digestion was stopped by addition of DFP at a 7 mM concentration. After 4 h, the mixtures were dialyzed against 1 mM HCl and lyophilized.

Equilibrium chromatography on CM-cellulose 32

The products resulting from the above-described incubation were fractionated by equilibrium chromatography on Whatman CM-cellulose 32 at pH 8.5 (buffer; 5 mM Tris and 100 mM acetate). The recommendations of Whatman for the removal of fines from CM-cellulose and for column packing were followed. In general, the procedure was very efficient and gave reproducible results.

Affinity chromatography for chymotrypsins

The active chymotrypsins arising from neochymotrypsinogen by incubation with trypsin were isolated by affinity chromatography in columns filled with Sepharose 4B coupled with aminocaproyl-D-tryptophan methylester[8]. This latter was prepared by condensation of D-tryptophan methylester with ε-carbobenzoxyaminocaproic acid. Various compounds strongly absorbing at 280 nm were removed from the eluate by extensive dialysis prior to spectroscopic determinations.

Active center titration by [^{32}P]DFP

The preparations containing chymotryptic activity (about 0.6 μmole) in 0.5 ml of 50 mM Tris buffer (pH 8.0) were incubated at 5°C for 3 h with 10 μl of a 0.4 M DFP solution containing 11.8 μCi of [^{32}P]DFP. The mixture was exhaustively dialyzed against water and the last traces of low molecular weight radioactive material were removed by a chromatography on CM-cellulose Whatman 32 under the conditions already described. A sample of chymotrypsin α was incubated simultaneously and the radioactive material was isolated by chromatography and counted in a Packard Tricarb Liquid Scintillation Spectrometer at the same time as the unknown sample. A 1:1 stoichiometric reaction between chymotrypsin α and DFP were assumed to take place.

End group determination

N-terminal residues in the preparations were identified and quantitated by the fluorodinitrobenzene (FDNB) method of Sanger. Ether-soluble dinitrophenyl (DNP)-derivatives were separated on paper by bidimensional chromatography (1st dimension: *tert.*-amyl alcohol–2 M ammonia (195:40, v/v), 48 h. 2nd dimension: 1 M sodium citrate buffer at pH 6.2, 18 h. C-terminal residues and sequences were ascertained by

digestion with carboxypeptidase A. The liberated amino acids were identified and quantitated with the aid of a Beckman amino acid analyzer Model 120 C.

RESULTS AND DISCUSSION

Digestion of chymotrypsinogen A with porcine chymotrypsin C

The N-terminal residues appearing in the protein fraction during the digestion of bovine chymotrypsinogen A with porcine chymotrypsin C in the presence of 1 M $(NH_4)_2SO_4$ are indicated in Table I. The Table also includes for purpose of comparison the residues generated by chymotrypsin α in the presence or absence of 1 M $(NH_4)_2SO_4$. The analyzed material also contained the N-terminal cystine pre-existent in native chymotrypsinogen A. This residue cannot be identified unless the protein is oxidized by performic acid.

TABLE I

N-TERMINAL RESIDUES APPEARING IN THE PROTEIN FRACTION DURING HYDROLYSIS OF CHYMOTRYPSINOGEN A BY CHYMOTRYPSIN C OR α

After incubation with DFP, dialysis and lyophilisation, the digests were condensed with FDNB. Small DNP-peptides and DNP-amino acids were washed by 1 M HCl, methylethyl ketone and ethanol, and the DNP-protein fraction was hydrolyzed. Ether-soluble DNP-amino acids were separated on paper. Uncorrected results are given in mole/mole of chymotrypsinogen A.

Chymotrypsin serving for hydrolysis:	*C*	*α*	*α*
Enzyme–substrate molar ratio	*0.15*	*0.10*	*0.10*
1 M $(NH_4)_2SO_4$	+	+	−
Threonine	0.1	0.1	0.5
Alanine	0.5	0.5	0.1
Serine	0.4	0.1	0.1
Others	0.1	<0.1	0.1

The results listed in Table I at first confirm the effect exerted by $(NH_4)_2SO_4$ (ref. 3) on the hydrolysis by chymotrypsin α of bond 148 responsible for the replacement of the N-terminal threonine (residue Thr_{147}) by a N-terminal alanine (residue Ala_{149}) in Sequence II. A most interesting observation is that chymotrypsin C appears to cleave in chymotrypsinogen A at least one bond which is not cleaved by chymotrypsin α. This additional hydrolysis resulting in the appearance of substantial amounts of N-terminal serine does not reduce the ability of the molecule to be activated later by trypsin. As a matter of fact, the specific potential activity towards ATEE of the chymotrypsin C digests is not significantly lower than that of similar digests by chymotrypsin α (480, 400, 250 and 200 after 0, 2, 27 and 48 h incubation, respectively). Although more degraded than the already known neochymotrypsinogens generated by chymotrypsin α, the new compound can therefore be expected to have equal ability to be activated.

Separation by chromatography of the new neochymotrypsinogen

In order to isolate and better characterize this compound, a digest of chymotrypsinogen A by chymotrypsin C was submitted to chromatography at pH 8.5 on a Whatman microgranular CM-cellulose 32 column. This type of column, previously

used for the fractionation of iodinated derivatives of chymotrypsinogen A (ref. 9), proved to be also quite efficient for the fine separation of the family of proteins derived from chymotrypsinogen A by limited proteolysis. At pH 8.5, any peptide bond cleavage in a protein can be expected to increase noticeably its negative charge, with the result that the most degraded derivative should emerge first from the column. Fig. 1a shows that chymotrypsin α, chymotrypsin π and chymotrypsinogen are eluted in this order by the pH 8.5 buffer. The additional separation of chymotrypsin δ from chymotrypsin π is due to the absence of residue Arg 15 in the first protein.

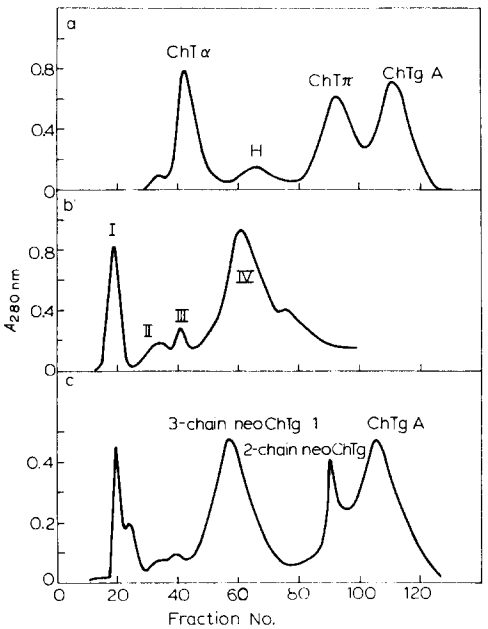

Fig. 1. Chromatography on CM-cellulose 32 at pH 8.5 of the family of proteins derived from chymotrypsinogen (ChTg) A by limited proteolysis. (a) The chymotrypsin (ChT) π sample containing some chymotrypsin δ was prepared by activation at 0 °C of bovine chymotrypsinogen A (15 mg/ml) by 0.75 mg/ml trypsin (enzyme–substrate molar ratio, 1:20) in the presence of 0.1 M β-phenylpropionate. After 90 min, the reaction was stopped by DFP added to a final concentration of 24 mM. The mixture was dialyzed against the pH 8.5 buffer containing 10 mM β-phenylpropionate. Chymotrypsinogen A and a commercial sample of chymotrypsin α (5 mg each) were added just before chromatography. The column (60 cm × 1.0 cm) was equilibrated and eluted by the same buffer at pH 8.5 (equilibrium chromatography). Elution rate: 5.6 ml/h. Volume of fractions, 1.4 ml. (b) Chymotrypsinogen A (60 mg) was incubated for 48 h at 5 °C with 9 mg of porcine chymotrypsin C. After dialysis and lyophilization, the powder was dissolved in 2 ml of the pH 8.5 buffer and the solution was introduced into a 60 cm × 2.0 cm column. Fractions 51–69 under Peak IV were pooled, dialyzed and lyophilized. (c) An aliquot (7 mg) of the lyophilized powder and chymotrypsinogen A (7 mg) were mixed with 7 mg of the product resulting from the digestion of chymotrypsinogen with chymotrypsin α (two-chain neochymotrypsinogen (neoChTg). Same chromatography as above in a 60 cm × 1.0 cm column.

The chromatographic diagram obtained with the digest of chymotrypsinogen A by chymotrypsin C is reproduced in Fig. 1b. Four well separated peaks are visible in this diagram. Peak I is a mixture of chymotrypsin C and inactive proteins. The proteins under Peaks II and III are also neither active nor are able to be activated. In contrast, the main Peak IV is inactive but can be activated and it can, therefore,

be assumed to correspond to the newly discovered neochymotrypsinogen. Accordingly, Fractions 51–69 under this peak were pooled, dialyzed and lyophilized.

Fig. 1c gives another example of the vast possibilities of CM-cellulose 32 chromatography for the fractionation of chymotrypsinogen A and its derivatives. The new neochymotrypsinogen, the already known neochymotrypsinogen with two chains and chymotrypsinogen A are seen to emerge in this order from the column and to give three well separated peaks. The early elution of the new neochymotrypsinogen confirms that this compound is more degraded than the 2-chain compounds. It will be proved later to contain 3 chains and is designated in Fig. 1 as 3-chain neochymotrypsinogen I. A still more degraded form (3-chain neochymotrypsinogen II) will be identified later.

Better characterization of neochymotrypsinogen I

The following end groups could be identified in the lyophilized powder prepared from Fractions 51–69 of Peak IV in Fig. 1b: N-terminal residues, alanine (0.4 mole/mole) and serine (0.3 mole); C-terminal residues, tyrosine (0.50 mole) and leucine (0.45 mole). To these residues, the N-terminal half cystine and the C-terminal asparagine pre-existing in native chymotrypsinogen A should be added.

The well-known lability of bonds 146 and 148 towards chymotrypsin α suggests that the same bonds are cleaved by chymotrypsin C. This assumption is further confirmed by the fact that a C-terminal arginine residue (Arg_{145}) appears in place of Tyr_{146} when the preparations of chymotrypsin C used for the digestion are contaminated by carboxypeptidase A. Consequently, the N-terminal alanine residue mentioned above is Ala_{149}.

The location of the N-terminal serine and the C-terminal leucine is more difficult to assess because of the presence of two leucyl–serine bonds (Leu_{10}–Ser_{11} and

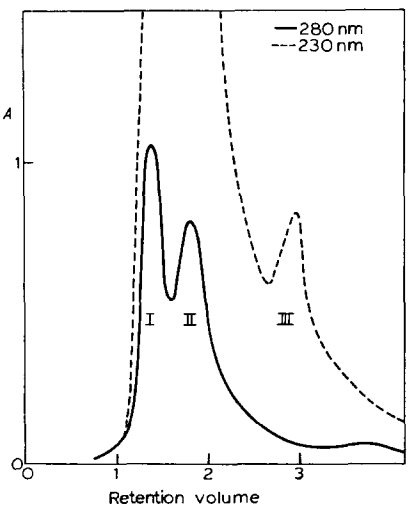

Fig. 2. Separation of the three chains of oxidized neochymotrypsinogen I. The enzyme precursor (30 mg) was oxidized by performic acid and lyophilized. The aqueous extracts of the lyophilized powder, after concentration at 0.5 ml were applied on a column (1 cm × 40 cm) of Sephadex G 50 "fine" and eluted with 5 mM HCl. Volume of fractions, 2 ml. Volume of retention, 12,4 ml. ———, absorbance at 280 nm; — — —, absorbance at 230 nm.

Leu_{13}–Ser_{14}; bonds 10 and 13) in the N-terminal regions of chymotrypsinogen A. Accordingly, the short chain arising by tryptic activation of neochymotrypsinogen I was separated by the same technique as for chymotrypsin α (ref. 10). This technique involves the liberation of the chains by performic acid oxidation and filtration of the water-soluble components through Sephadex G-50. The results obtained are illustrated in Fig. 2. As in the case of chymotrypsin α, the material under the most retarded peak (Peak III) had no absorbance at 280 nm, but absorbed strongly at 230 nm. After purification by electrophoresis–chromatography on paper, a peptide with the following composition was obtained: cysteine sulfonic acid (1.0), glutamic acid (1.1), proline (1.9), glycine (1.1), alanine (1.1), valine (1.9), isoleucine (1.0), leucine (0.9). This composition is identical to that of the 10 first residues of the N-terminal sequence of bovine chymotrypsinogen A. The conclusion, therefore, is that neochymotrypsinogen I contains a 10-residue chain beginning with residue Cys_1 and ending with residue Leu_{10}. Quite obviously, chymotrypsin C has been able to split in Sequence I either bond 10 or bonds 10 and 13. That both bonds 10 and 13 are actually cleaved was demonstrated by the fact that approximately stoichiometric amounts of the dipeptide Ser–Arg (arising from residue Ser_{14} and Arg_{15}) were liberated upon activation of neochymotrypsinogen I by trypsin in the presence of β-phenyl propionate. Neochymotrypsinogen I contains three chains which are: Cys_1–Leu_{10}; Ser_{14}–Tyr_{146} and Ala_{149}–Asn_{245}.

Activation of the three-chain neochymotrypsinogen I

A three-chain neochymotrypsinogen I solution (30 mg/ml) in a 50 mM Tris buffer (pH 8.0) was incubated at 0°C with 0.75 mg/ml of trypsin. The specific activity (ATEE) of the mixture reached a maximal value of 220 after 120 min. At this point, the solution was dialyzed against water and lyophilized. An aliquot (5 mg) of the lyophilized powder was purified by affinity chromatography in a 1.0 cm × 2.5 cm column of Sepharose 4 B coupled with ε-aminocaproyl-D-tryptophan methylester[8]. A preliminary washing of the column with a 50 mM Tris buffer (pH 8.5) removed approx. 0.8 mg of inert proteins. Then, elution with 0.1 M acetic acid induced the emergence of 2.7 mg of a chymotrypsin (chymotrypsin I) with a specific activity (ATEE) of 350. Under the same conditions, the specific activity of a commercial sample of chymotrypsin α was 420 before and 450 after affinity chromatography. In both purified preparations, a titration with the aid of [^{32}P]DFP indicated the presence of one active site per mole. Table II shows that the kinetic parameters of the hydrolysis of ATEE and ATA by the new chymotrypsin and by chymotrypsin α are approximately the same.

Since chymotrypsin I and chymotrypsin α differ from each other by the absence

TABLE II

KINETIC PARAMETERS OF REACTIONS CATALYZED BY CHYMOTRYPSIN I AND BY CHYMOTRYPSIN α

Substrate	*V (min^{-1})*		*K_m (mM)*	
	New chymotrypsin	*Chymotrypsin α*	*New chymotrypsin*	*Chymotrypsin α*
ATEE	350	450	0.94	0.83
ATA	$1.64 \cdot 10^{-6}$	$2.1 \cdot 10^{-6}$	34	53

in the first of residues Ser_{11}, Gly_{12} and Leu_{13}, it can be concluded that the loss of these residues does not alter significantly the catalytic power of the molecule.

Conversion of neochymotrypsinogen I into neochymotrypsinogen II by splitting off the residues Tyr_{146} and Arg_{145}

It has already been pointed out that some preparations of porcine chymotrypsin C were slightly contaminated by carboxypeptidase A activity and that these preparations split off the C-terminal Tyr_{146} residue in neochymotrypsinogen I. The preceding residue Arg_{145} can subsequently be detached in a selective manner by carboxypeptidase B, leaving a second neochymotrypsinogen (neochymotrypsinogen II) with 2 residues less than neochymotrypsinogen I.

The neochymotrypsinogen lacking residue Tyr_{146} (50 mg) was dissolved in 3 ml of 1 mM HCl and mixed with a solution of 50 μl of 0.4 M DFP in 7 ml of a 0.2 M triethylamine–carbonate buffer (pH 9.0). DFP-treated carboxypeptidase B (1.2 mg in 250 μl) was added and the incubation was pursued for a total of 24 h at 0°C with a second addition of enzyme at 6 h. At the end of the incubation, an aliquot (0.5 ml) of the mixture was removed, acidified at pH 3.0 with a few drops of acetic acid, evaporated under reduced pressure and analyzed for free amino acids. No amino acid other than arginine (0.6 mole/mole, not corrected) could be identified. The remainder of the digest was then acidified as above, dialyzed and lyophilized. Fig. 3 indicates

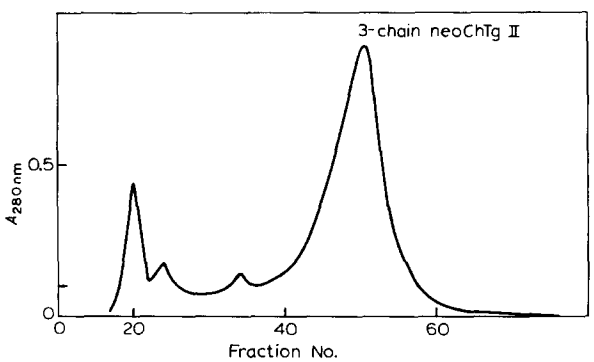

Fig. 3. Chromatography on CM-cellulose 32 of neochymotrypsinogen (neoChTg) II. The conditions under which the chromatography was performed are the same as in the experiments illustrated by Fig. 1.

the diagram obtained when the product thus obtained was chromatographed in a CM-cellulose column at pH 8.5. The main component was eluted distinctly faster than neochymotrypsinogen I, an observation consistent with the loss of one arginine residue. In addition, Table III shows that neochymotrypsinogen II contains one arginine as well as one tyrosine less than native chymotrypsinogen A.

An interesting point is that neochymotrypsinogen II is normally activatable by trypsin. After an incubation with trypsin performed as for neochymotrypsinogen I, the resulting chymotrypsin (chymotrypsin II) was purified by affinity chromatography and found to have a specific activity towards ATEE as high as 350.

It seems, therefore, well established that the cleavage of bonds 10 and 13 and

TABLE III

AMINO ACID COMPOSITION OF NEOCHYMOTRYPSINOGEN II AND CHYMOTRYPSIN II

The results are given in moles of residues per mole of protein.

Residue	*Neochymotrypsinogen II*	*Chymotrypsinogen A*	*Chymotrypsin II*	*Chymotrypsin α*
Arg	*3.2*	*4*	*2.2*	*3*
His	1.9	2	1.9	2
Lys	14.4	14	13.9	14
Met	1.9	2	1.9	2
Phe	6.0	6	5.8	6
Tyr	*3.1*	*4*	*3.1*	*4*

the release of residue Tyr_{146} and Arg_{145} are compatible with chymotrypsinogen activation and chymotryptic activity. The fact that Tyr_{146} is not essential for chymotrypsin activity has already been reported[11]. In contrast, the ability of neochymotrypsinogen II lacking residue Arg_{145} to be activated is especially noteworthy. A repositioning of the guanidinium group of this residue 15 Å away from its original position in the zymogen has recently been observed by Freer *et al.*[12] with the aid of crystallographic techniques. This observation and the striking conservation of a basic residue in position 145 in bovine trypsinogen, bovine chymotrypsinogen A and B, porcine elastase and bovine thrombin led to the assumption that Arg_{145} might help Asp_{194} to move away from His_{40} towards Ile_{16}. The isolation of an active chymotrypsin II is not consistent with this assumption.

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REFERENCES

1 P. Desnuelle and M. Rovery, *Adv. Protein Chem.*, 16 (1961) 139.
2 M. Rovery, M. Poilroux, A. Curnier and P. Desnuelle, *Biochim. Biophys. Acta*, 17 (1955) 565.
3 M. Rovery, M. Poilroux, A. Yoshida and P. Desnuelle, *Biochim. Biophys. Acta*, 23 (1957) 620.
4 F. R. Bettelheim and H. Neurath, *J. Biol. Chem.*, 212 (1955) 241.
5 W. H. Dreyer and H. Neurath, *J. Biol. Chem.*, 217 (1955) 527.
6 O. Guy, D. Gratecos, M. Rovery and P. Desnuelle, *Biochim. Biophys. Acta*, 115 (1966) 404.
7 D. Gratecos and P. Desnuelle, *Biochem. Biophys. Res. Commun.*, 42 (1971) 857.
8 P. Cuatrecasas, M. Wilchek and C. B. Anfinsen, *Proc. Natl. Acad. Sci. U.S.*, 61 (1968) 636.
9 B. H. Weber and J. Kraut, *Biochem. Biophys. Res. Commun.*, 33 (1968) 272.
10 Dinh Van Hoang, M. Rovery and P. Desnuelle, *Biochim. Biophys. Acta*, 58 (1962) 613.
11 J. A. Gladner and H. Neurath, *J. Biol. Chem.*, 206 (1954) 911.
12 T. Freer, J. Kraut, J. D. Robertus, H .T. Wright and N. G. H. Xuong, *Biochemistry*, 9 (1970) 1997.