STRUCTURAL CHANGES ASSOCIATED WITH THE CONVERSION OF PEPSINOGEN TO PEPSIN*

II. THE N-TERMINAL AMINO ACID RESIDUES OF PEPSIN AND PEPSINOGEN; THE AMINO ACID COMPOSITION OF PEPSINOGEN

HELEN VAN VUNAKIS** AND ROGER M. HERRIOTT

Department of Biochemistry, Johns Hopkins University, School of Hygiene and Public Health, Baltimore, Md. (U.S.A)

The amino acid composition and N-terminal amino acid residue of the pepsin inhibitor obtained during the conversion of pepsinogen into pepsin were given in an earlier publication[2]. Similar information is now presented for pepsinogen and pepsin.

The finding of different, single N-terminal residues in both proteins suggests that the molecules are composed of single peptide chains and that pepsin does not occupy the N-terminal position in the precursor, pepsinogen. This was also shown to be true for the inhibitor from studies of the amino acid sequence near the N-terminal end.

The amino acid composition of pepsinogen was determined using the chromatographic methods developed by MOORE AND STEIN[3,4]. This data, together with the amino acid analysis of pepsin[5] and the inhibitor[2], make it possible to obtain an approximation of the amino acids present in the "miscellaneous" peptides formed during the conversion of pepsinogen into pepsin.

MATERIAL AND METHODS

Protein and peptide preparations

Exhaustively dialyzed samples of swine pepsinogen[6] and swine pepsin fractionated as outlined in[7] have been used in this work. Swine pepsin obtained from Worthington Biochemical Corporation (Lot # PMA 525) was used to check the results of one experiment. Activation of pepsinogen by acidification was performed as described earlier[8,9]. The TCA-soluble material was separated into acidic (A), neutral (N), and basic (B) fractions using ion-exchange resins[2].

The fundamentals of the DNP technique have been thoroughly described[10,11,12]. Therefore, only those experimental procedures will be discussed which differ from those considered in these references.

Preparation of DNP proteins and DNP peptides

DNP pepsinogen was prepared by shaking 200 mg of protein with 600 mg of dinitrofluorobenzene (DNFB) in an alcohol-aqueous bicarbonate solution for 2 hours. DNP pepsinogen precipitated during the course of the reaction. The derivative was centrifuged, washed with water, alcohol, and ether, and dried over P_2O_5 *in vacuo*.

DNP pepsin was obtained in a similar manner, using 200 mg pepsin in 10% $NaHCO_3$ with 600 mg DNFB in alcohol. However, its solubility was somewhat variable. When the reaction took

* The results of these studies were summarized in an article published earlier[1].

** Public Health Service Research Fellow of the National Cancer Institute. Present address: Laboratories of Biochemistry and Immunology, Division of Laboratories and Research, New York State Department of Health, Albany 1, New York.

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place in 3% $NaHCO_3$ solution instead of the usual 10%, a water-soluble derivative was obtained. This was dialyzed against H_2O to remove excess reagents and then lyophilized. In another case, DNP pepsin was insoluble in water but soluble in alcohol. It was precipitated by addition of ether to the alcohol solution. The picture is complicated somewhat by the fact that pepsin is rapidly denatured at the alkaline pH of the bicarbonate solution.

EXPERIMENTAL RESULTS

Identification and estimation of N-terminal amino acids of pepsinogen and pepsin

Using the DNP derivatives of pepsin and pepsinogen, hydrolysis, fractionation, and identification of N-terminal amino acids was undertaken following the methods of SANGER[10]. Samples of the DNP derivatives of the two proteins were hydrolyzed in 6 *N* HCl as described in Table I and the hydrolysates extracted with ether. The latter step separates the DNP amino acids into (a) the acid fraction which may contain bis-DNP histidine, -S DNP cysteine, DNP arginine, ε-DNP lysine plus α-DNP lysine, and (b) the ether fraction which may contain any of the other DNP amino acids. These fractions were concentrated to dryness, *in vacuo*, and the residues were chromatographed on silica-gel columns[10] employing a variety of solvent systems[10–13]. Final identification depended on separating the isolated DNP amino acid from all the other known DNP amino acids except the suspected one. The DNP amino acids were also chromatographed on paper using butyl alcohol saturated with water containing ammonia[12]. The results were confirmed by decomposing the DNP amino acid with either $Ba(OH)_2$[14] or NH_4OH[15] and identifying the free amino acid by paper chromatography.

DNP pepsinogen and DNP pepsin were subjected to several hydrolytic conditions so that those DNP amino acids (proline, hydroxyproline, and glycine) which are labile to the usual treatment in 6 *N* HCl could be detected if they were present as terminal amino acids[10,13]. Neither DNP proline nor DNP hydroxyproline were detected when the hydrolysis was carried out under conditions known to preserve these derivatives, *i.e.*, hydrolysis for 16 hours in 12 *N* HCl at 105°C. DNP glycine was absent in those samples that were hydrolysed for 4 hours in 6 *N* HCl at 105°C.

When the ether extracts of the DNP pepsinogen and DNP pepsin hydrolysates were chromatographed on a $CHCl_3$–H_2O silica gel column, a fast-running decomposition band plus a main band that had an R_F of approximately 0.45 was obtained in each case. This band was not split by further fractionation on columns using the organic phase of acetone–cyclohexane–H_2O (1:10:1) ($R_F = 0.6$), or ethanol–ligroin–H_2O (1:10:1) ($R_F = 0.8$) as developing solvents. Synthetic DNP leucine and DNP isoleucine were the only derivatives that had corresponding R_F values in all these solvents. The isolated products were also chromatographed on paper using butyl alcohol–water–ammonia as the solvent system and were found to fall into the same group as synthetic DNP leucine or isoleucine[12]. Thus far, the separation of DNP leucine and DNP isoleucine has not been successful. Therefore, alkaline hydrolysis was employed to obtain the free amino acid which was identified by paper chromatography using butyl alcohol–water–acetic acid (4:5:1) and phenol saturated with water. The R_F values obtained again indicated one of the leucines. A satisfactory separation of these isomers was achieved by using *tert.*-amyl alcohol–water and running a descending paper chromatogram for 6–7 days[16].

The amino acid thus identified as the N-terminal residue of pepsinogen was leucine, while isoleucine was found on the corresponding position of pepsin*. The latter was identified in commercial (Worthington) pepsin as well as that produced by the activation of pepsinogen.

WILLIAMSON AND PASSMAN[19,20] using the DNP technique reported that the N-terminal group of pepsin is leucine. They resolved their hydrolysate on a two-dimensional paper chromatogram using *m*-cresol-0.3% NH_4OH and collidine as solvents. There is some question whether this solvent combination has the resolving power of *tert.*-amyl alcohol[16] for the leucine isomers.

Since there was some decomposition of DNP leucine and DNP isoleucine during the acid hydrolysis required to split the peptide bonds of the DNP proteins, the syn-

* INGRAM's work[17] on autolyzed pepsin appears to have been misinterpreted[18]. This work does not provide any information about the N-terminal amino acid of the enzyme, but rather of some autolyzed fragments of low molecular weight.

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thetic DNP amino acids were subjected to hydrolysis in 6*N* HCl for varying periods of time and the amount destroyed estimated colorimetrically. When the values for the isolated DNP amino acids were corrected accordingly, the results showed that only one mole of terminal amino acid is present per mole of pepsinogen and pepsin (Table I). The calculations were carried out assuming a molecular weight of 34,500 for pepsin and 42,500 for pepsinogen. These represented average values of several different determinations. The molecular weight of pepsin has been obtained by osmotic pressure[21,22], ultracentrifuge diffusion[23,24,25], amino acid analysis[5], P analysis[26], and spread monolayers[27,28], while that of pepsinogen has been obtained by osmotic pressure and light scattering[29].

TABLE I

NUMBER OF TERMINAL AMINO ACID PER MOLE OF PROTEIN

Preparation	*Hours of hydrolysis in 6 N HCl*	*mg DNP protein*	*mg Terminal DNP group isolated*	*mg Terminal DNP group corrected for hydrolysis*	*% Terminal DNP group*	*Number of terminal residues per mole of protein*
DNP pepsin*	4	108	0.328	0.39	0.36	0.85
	15	145	0.397	0.58	0.40	0.93
	18	118	0.330	0.49	0.42	0.98
DNP pepsinogen**	4	93	0.210	0.25	0.27	0.72
	16	140	0.400	0.57	0.40	1.2
	18	95	0.274	0.41	0.43	1.1

* M.W. = 34,500. The N-terminal amino acid is isoleucine.
** M.W. = 42,000. The N-terminal amino acid is leucine.

The finding that the N-terminal amino acids of the two proteins were different indicates that pepsin does not occupy the N-terminal position in the structure of pepsinogen.

The presence of only one N-terminal residue in both pepsin and pepsinogen suggests but does not prove that these proteins are composed of single peptide chains. The reduction studies of KERN[22] provide additional evidence to support this view. He found no decrease in the molecular weight of pepsin and pepsinogen when the three disulfide bridges in these proteins were completely reduced, indicating that these bridges do not unite separate peptide chains. There was also no loss in nitrogen on dialysis of the reduced products, showing that the disulfide bridges are not linking small peptides (whose loss might not be detected by molecular weight determinations) to the main protein branch.

Amino acid sequence in the N-terminal segment of pepsinogen, pepsin and the inhibitor

The N-terminal amino acid of the inhibitor was found to be leucine[2] and the corresponding position in pepsinogen and pepsin was found to be occupied by leucine and isoleucine respectively. It was necessary, therefore, to determine the second and perhaps the third amino acid in pepsinogen and the inhibitor in order to decide if the inhibitor occupies the terminal position in the precursor. In view of the finding of WILLIAMSON AND PASSMAN[20] that the terminal group is pepsin in leucine, this protein was likewise included in the study. The results are shown in Table II.

The sequence of amino acids was obtained by digesting the DNP derivatives for short intervals with 6 *N* HCl and removing the DNP amino acids and DNP peptides by

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ethyl acetate extraction. Fractionation was carried out on a series of butanol-$CHCl_3$/silica gel columns. The homogeneous bands obtained were completely hydrolyzed in 6 *N* HCl and the free amino acids identified on paper chromatograms using BuOH·HAc·H_2O (40:10:50) and phenol saturated with water as solvents. The risk in this type of procedure is that rearrangements of amino acids during hydrolysis may occur[10]. However, SANGER AND THOMPSON found an inversion in peptide sequence during hydrolysis in dilute acid to be more prevalent than in concentrated solution[30].

TABLE II

N-TERMINAL AMINO ACID SEQUENCE OF PEPSINOGEN, PEPSIN, AND THE INHIBITOR

	N-terminal amino acid	*Second amino acid*
Pepsinogen	Leucine	Isoleucine or leucine
Pepsin	Isoleucine	Glycine
Inhibitor	Leucine	Glutamic acid

It was found that the N-terminal amino acid sequence of neither pepsin nor the inhibitor matched the sequence found in pepsinogen. The TCA-soluble components were not examined. Using a modification of Edman's degradative method PASSMAN AND WILLIAMSON[32] have identified the N-terminal sequence in pepsin as leucyl–glycyl–aspartyl–aspartyl–. We had identified glycine as the second amino acid[1], but as noted earlier, we differ on the identity of the terminal amino acid.

The lysine residues

Only ε-DNP lysine was detected in the acid fraction of the hydrolysates of DNP pepsinogen and DNP pepsin. Eleven lysine residues were present per mole of pepsinogen while two were found in pepsin (Table III). Four are located in the inhibitor[2]. The lysine value obtained for pepsin agrees with that previously found by an independent method[5]. Microbiological assay also revealed the presence of eleven lysine residues per mole of pepsinogen. Amino acid analysis of this protein hydrolysate after separation on Dowex-50-X4 indicated the presence of twelve lysine residues. Therefore, unlike some other native proteins that have been studied[10,11], the ε-amino groups of lysine in pepsinogen were all available for reaction for DNFB. This was also true for pepsin, but in this instance the protein is denatured at the pH of the

TABLE III

NUMBER OF ε-DNP LYSINE RESIDUES PER MOLE OF PROTEIN

Preparation	*Hours of hydrolysis in 6 N HCl*	*mg DNP protein*	*mg DNP from ε-DNP lysine*	*mg DNP corrected for decomposition*	*% DNP*	*Number of lysine residues per mole protein*
DNP pepsin	18	118	0.85	0.95	0.81	1.9
	24	64	0.369	0.41	0.64	1.6
	24	80	0.496	0.58	0.73	1.7
DNP pepsinogen	18	95	3.31	3.68	3.98	10.6
	24	70	2.83	3.32	4.25	11.2

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reaction mixture and the ε-amino groups of denatured proteins are usually available for reaction with DNFB.

Van Slyke-NH_2N determinations had previously indicated the presence of about 5 free amino groups in pepsin and 17 in pepsinogen[32]. Differences between the number of free amino groups obtained by the nitrous acid procedure and the DNP technique were also noticed in the case of the serum albumins[12] and several other proteins[10,13]. In general, the Van Slyke determination seems to give higher values for proteins than that found by end group methods.

TCA-soluble components

The TCA-soluble components of the activation mixture include the inhibitor of pepsin and a number of miscellaneous peptides[9]. These have been separated by means of an ion-exchange process[2] into basic (B), neutral (N), and acidic (A) fractions. The basic fraction contained all the inhibitor and was at least 85% as pure as the crystalline standard preparation[9]. This fraction has already been examined[2]. Fraction A and N were also treated with DNFB and the results summarized in Table IV.

A number of things are of interest. First, the DNP reaction with the A fraction was so slight that the calculated minimum molecular weight was high and unreasonable in view of the fact that pepsin constitutes 80% of pepsinogen. This fraction is probably degraded pepsin which is soluble in TCA. It represented only 3.9% of the total pepsinogen nitrogen.

The N fraction, constituting nearly 12% of the pepsinogen nitrogen, is equivalent

TABLE IV

ANALYSIS OF PEPSINOGEN ACTIVATION MIXTURE BY DNP METHOD

Molecular weight pepsinogen 42,500 (Os. press., light scattering, P analysis)
Molecular weight pepsin 34,500 (Os. press., centrifugation, amino acids, S and P analysis)
Difference 8,000 = 19% of pepsinogen

Net increase in NH_2–N (Van Slyke) on activation = 9 groups/mole pepsinogen
Activation of 4 g pepsinogen at pH 2 for 1 min, then fractionated

Fraction	*Percentage of pepsinogen N*	*NH_2-N as percentage of total N*	*Minimum mol. wt.*	*No. of α-NH_2 peptides**	*Total mol. wt.*
Pepsinogen (before activation)	100.0		40,000	1	42,500
Soluble in 2½% TCA					
Basic (inhibitor)	6.3		3,100	1**	3,100
Acidic	3.9	0.5	20,000(?)	0	0
Neutral	11.9	10.0	1,000	5	5,000
Total TCA-soluble	22.1			6	8,100
Pepsin	77.8		41,000	1	34,500
Total activation mixture	99.9			7	42,600

* This figure was obtained in different ways. For pepsinogen and pepsin the known molecular weight of the protein was divided by the minimum molecular weight calculated from the DNP analysis (see data in Table I) and the final figure rounded out to the nearest integer. For the basic, acidic, and neutral fractions of the TCA-soluble material, the figure was obtained by multiplying 42,500 by the percentage of pepsinogen nitrogen in the fraction and dividing by the minimum molecular weight derived from the DNP analyses. This procedure assumes the percentage nitrogen in the fraction is the same as in pepsinogen.

** The N-terminal analyses were performed on a DNP-inhibitor preparation that had been extracted with methyl ketone to remove small amounts of contaminating peptides[2].

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to a structure of 5000 molecular weight if the nitrogen content is the same as in pepsinogen. Five groups were found on DNP analysis indicating that the peptides present have an average minimum molecular weight of 1000.

Exclusive of the acidic fraction these DNP analyses account for a net increase of 7 terminal nitrogens as NH_2 groups. An increase of nine groups had been found by direct analysis of the activation mixture using the nitrous acid manometric method of Van Slyke. Although there are many bonds opened during conversion of pepsinogen to pepsin only one of these bonds may be essential to the process, the other cleavages being secondary effects.

Amino acid composition of pepsinogen

BRAND[5] made a nearly complete amino acid analysis of purified pepsin a number of years ago, and an analysis of the isolated inhibitor was reported recently[2]. Using the chromatographic methods developed by MOORE AND STEIN[3,4] the amino acid composition of a pepsinogen preparation was determined. The results are shown in Table V.

TABLE V

AMINO ACID COMPOSITION OF PEPSINOGEN*

Amino acid	*Grams of amino acid per 100 g protein***	*Grams of nitrogen per 100 g protein*	*Residues mole per mole protein (corrected to the nearest integer)*
Aspartic acid	14.6	1.53	46
Glutamic acid	11.1	1.16	32
Glycine	6.5	1.22	36
Alanine	5.8	0.91	27
Valine	7.6	0.91	27
Leucine	20.0	2.14	64
Isoleucine			
Serine	13.4	1.78	53
Threonine	7.7	0.90	25
Half-cystine	(1.1)***	(0.13)	(6)
Methionine	(1.1)	(0.10)	(5)
Proline	5.6	0.68	20
Phenylalanine	8.0	0.68	20
Tyrosine	7.0	0.54	16
Tryptophan	(1.9)	(0.13)	(4)
Histidine	1.4	0.38	4
Lysine	4.2	0.81	12
Arginine	1.3	0.42	3
Amide NH_3	(1.6)	(1.30)	
Total	119.9	15.7	400

* These analyses were performed at the Division of Laboratories and Research, New York State Department of Health.

** The average per cent difference for the amino acids determined by four chromatographic analysis was 7.8%.

*** The figures in parentheses were not determined directly. See text for a discussion.

For purposes of comparison the amino acid composition of pepsin and the inhibitor are included with the pepsinogen figures in Table VI. From these data an approximation of the amino acids in the "miscellaneous peptides" produced during conversion of pepsinogen can be obtained for they represent the difference between pepsinogen and the sum of pepsin and the inhibitor.

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TABLE VI

AMINO ACID COMPOSITION OF PEPSINOGEN, PEPSIN AND THE INHIBITOR

Amino acid	Residue weight	Residues per mole*				Total residues weights or residue weight number/mole		
		Pepsinogen 1	Pepsin 2	Inhibitor 3	Misc. peptides 1—(2 + 3)	Pepsinogen	Pepsin	Inhibitor
Aspartic acid	115	46	41	4	1	5290	4600	460
Glutamic acid	129	32	28	2	2	4125	3615	258
Glycine	57	36	29	1	6	2050	1652	57
Alanine	71	27	(21)**	2	4	1920	1490	142
Valine	99	27	21	2	4	2675	2080	198
Leucine	113	64	27	5	4	7230	6215	565
Isoleucine	113		28					
Serine	87	53	40	2	11	4660	3520	174
Threonine	101	25	28	1	0	2530	2830	101
Half-cystine	102	(6)	5+1		0	612	612	
Methionine	131	(5)	4		0	393	524	
Proline	97	20	15	3	2	1940	1455	291
Hydroxyproline	—	—	0	—	—			
Phenylalanine	147	20	13	1	6	2940	1910	147
Tyrosine	163	16	16	1	0	2610	2610	163
Tryptophan	186	(4)	4			744	744	
Histidine	137	4	2		2	548	274	
Lysine	228	12	2	4	6	1536	256	512
Arginine	156	3	2	1	0	468	312	156
Amide NH_3	16	(39)	32			512	512	
PO_3						79	79	
Total amino acid residues/mole		400	327	29	44–48			
Sum of amino acid residue weights						42,862	35,298	3,242
Molecular weight (physical or chemical methods)						42,500	34,500	3,100

* Rounded off to the nearest integer.
** The figures in parentheses were not determined directly. See text for discussion.

Six to ten mg of pepsinogen was hydrolyzed in 6 *N* HCl for 37 to 72 hours in a sealed tube at 105°C *in vacuo*. After removal of the HCl over KOH, the residue was dissolved, adjusted to pH 2 and the amino acids separated on a Dowex-50-X4 column according to the procedures of MOORE AND STEIN[3]. Only a minor change was made from the procedure described by these authors. The initial temperature of the chromatographic run was set at 35°C instead of the recommended 30°C. This gave better resolution of the aspartic, threonine, and serine peaks with the particular batch of resin that we used. The separation between leucine and isoleucine was not good enough to warrant assigning individual values to these two amino acids; therefore, one value is given for both.

The cystine and methionine content were obtained from the data of KERN[22] who found 0.82% sulfur or 11 sulfurs per mole pepsinogen whereas for pepsin the number was 10. By an independent assay[33] KERN found 3 cystines or 6 half cystines in pepsinogen and the difference of 5 sulfurs in pepsinogen is assumed to be methionine. BRAND found 4 methionines in pepsin[5] and it is tentatively assumed that the additional sulfur atom in pepsinogen is in methionine.

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The tryptophan of pepsinogen was assumed to be the same as in pepsin. No direct analyses were performed.

Corrections for decomposition of several amino acids have been made in accordance with the findings of other workers in the field[34,35]. These corrections amounted to 5% for aspartic acid, threonine, and tyrosine while a 10% correction was necessary for serine. The analyses in Table V represent the average of four complete chromatographic separations.

BRAND[5] did not determine alanine in pepsin. In Table VI it has been assumed that the alanine of pepsinogen was evenly distributed over the entire molecule. BRAND also reported one cysteine and five half cystines from pepsin. KERN[22] found 6 half cystines in both pepsin and pepsinogen by the ANSON procedure[33], so that for accounting purposes the half cystines in pepsin are placed at 6. The amide nitrogen is taken from the analyses on pepsin.

The most striking difference about the amino acid composition of pepsinogen is that there is considerably more lysine in the precursor than in pepsin. However, pepsin is unusually low in basic amino acids.

If the various corrections and inclusions that have already been discussed are accepted, a few points take on some interest. The sums of the total amino acid residue weights shown in Table VI are in close agreement with the molecular weight values obtained independently by a variety of methods. This was true for pepsin and the inhibitor as well as pepsinogen.

It was also possible to obtain the composition of the trichloracetic acid-soluble fragments other than the inhibitor as shown at the extreme right side of Table VI in the column denoted 1—(2+3). The value represents the number of amino acid residues in pepsinogen minus the sum of the numbers in pepsin and the inhibitor. This TCA-soluble fraction is mainly the neutral fraction previously described[2] and consists of 5 peptides having an average molecular weight of 1000.

SUMMARY

1. The N-terminal amino acids of pepsinogen, pepsin, and the inhibitor were determined using the dinitrophenol technique of SANGER and found to be leucine, isoleucine, and leucine respectively.

2. The sequence of amino acids at the N-terminal position of pepsinogen, pepsin, and the inhibitor are different; hence, neither pepsin nor the inhibitor can occupy the N-terminal position in pepsinogen.

3. The precursor, enzyme, and inhibitor were each found to have one N-terminal residue, indicating that these molecules are composed of single peptide chains.

4. The ε-amino groups of the eleven lysines of pepsinogen and the two in pepsin are free to react with dinitrofluorobenzene.

5. A DNP analysis of the neutral fraction of the TCA-soluble components of the activation mixture shows that it consists of five peptides having an average molecular weight of 1000.

6. The amino acid composition of pepsinogen has been determined using the ion-exchange resin, Dowex-50-X4. It contains considerably more lysine than pepsin. The sum of the residue weights agrees with the molecular weight obtained by chemical and physical methods.

7. The amino acid composition of the remainder of pepsinogen (miscellaneous peptides) was determined by subtracting the amino acid composition of pepsin and the inhibitor from pepsinogen.

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SUR LA DÉGRADATION DU CHYMOTRYPSINOGÈNE PAR LA CHYMOTRYPSINE

M. ROVERY, M. POILROUX, A. YOSHIDA* ET P. DESNUELLE
Laboratoire de Chimie biologique, Faculté des Sciences, Marseille (France)

L'attaque du chymotrypsinogène par la trypsine a été récemment étudiée de façon précise[1-5]. On sait qu'elle est rapide, même à basse température, et qu'elle a pour effet principal de rompre une liaison peptidique reliant un résidu d'arginine à un résidu d'isoleucine (liaison (1)). On sait également que cette rupture provoque l'activation du zymogène c'est à dire sa transformation en chymotrypsine-π, premier représentant de la famille des chymotrypsines. L'enzyme-π s'autolyse ensuite au

* Professeur associé de l'Université de Tokyo.

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