PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

# Chemical Studies of Porcine Pepsin

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# II. ACID PROTEINASES

## Chemical studies of porcine pepsin

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I was asked by the organizers of this meeting to summarize what has been known up to now about the chemistry of pepsin. My task was difficult for several reasons. It is not easy to discuss a subject of which our knowledge is relatively meagre after such excellent talks as the ones on pancreatic enzymes this morning; later I learned more about the schedule and found out that the most interesting part of the chemistry of pepsin, namely its proteolytic activity, will be treated by the next speaker. But then I did a little bit of calculation and found, first, that the data already published on the primary structure of pepsin contain about 63 % of its residues in known sequences, and secondly, taking in mind that I came from Prague by car, that each known peptide bond in pepsin is equal to about 16 km of hard drive, Channel not included. Both these facts are good reasons why I should try my best to summarize for you the primary structure of pepsin.

To adhere to tradition and to take the orthodox historical approach to the subject, I have to draw your attention to the fact that pepsin is probably the first material in which enzymic activity was described—by Spalanzani 185 years ago. Its precursor, pepsinogen, was discovered only some 100 years later.

Although porcine pepsin was isolated in pure state earlier than trypsin or chymotrypsin (Kunitz & Northrop 1930) studies of its structure were handicapped predominantly for two reasons: its preparation in pure state was difficult and its amino acid composition was far from being an ideal one for chemical studies. As regards the purity of pepsin, this question was probably definitely solved by the work of Rajagopalan, Stein & Moore (1966), who have shown that pepsin prepared from crystalline pepsinogen by short activation under strictly controlled conditions conforms to all criteria for a homogeneous protein.

Table 1 shows the amino acid composition of pepsinogen and pepsin as determined by the Rockefeller group. In addition to the data presented in this table an additional characteristic

Table 1. Amino acid composition of porcine pepsinogen and pepsin (residues per molecule)

| amico acid | pepsinogen | pepsin | difference | amino acid | pepsinogen | pepsin | difference |
|---|---|---|---|---|---|---|---|
| Lys | 10 | 1 | 9 | Ala | 19 | 16 | 3 |
| His | 3 | 1 | 2 | Val | 23 | 20 | 3 |
| Arg | 4 | 2 | 2 | Cys | 6 | 6 | 0 |
| Asp | 44 | 40 | 4 | Met | 4 | 4 | 0 |
| Thr | 26 | 25 | 1 | Ile | 25 | 23 | 2 |
| Ser | 46 | 43 | 3 | Leu | 33 | 28 | 5 |
| Glu | 28 | 26 | 2 | Tyr | 17 | 16 | 1 |
| Pro | 19 | 16 | 3 | Phe | 15 | 14 | 1 |
| Gly | 35 | 34 | 1 | Trp | 6 | 6 | 0 |
| | | | | total | 363 | 321 | 42 |

of pepsin should be mentioned: the presence of phosphate group which is bound to one of the 43 serine residues and which contributes to the extremely low isoelectric point of pepsin. As you can see the most striking difference between pepsinogen and pepsin is in the content of basic amino acids. When pepsinogen is activated to pepsin, the majority of the basic amino acids are split off as the so-called activation peptides, leaving in the protein only two arginines, one lysine and one histidine.

Because both pepsinogen and pepsin are single chain molecules, the task of elucidation of the primary structure is to determine the sequence of 363 and 321 amino acid residues respectively. There are two approaches—either to look for sequences around the most characteristic amino acid residues or to try to get a limited number of large fragments. Both pathways have been followed in several laboratories.

Let us examine first the amino acid composition and what comes out as an attractive point of attack. If there were a favourable distribution of the two arginines and one lysine along the chain, a tryptic cleavage would yield four nice fragments. Unfortunately, the fragments are not nice, because all these three basic residues are within the last twenty residues at the carboxyl end of the molecule, as we will see later. The next least abundant amino acid is cysteine. There are six cystine residues, forming three disulphide bridges whose relative position is interesting from the aspect of spatial arrangement of the chain. There are four methionine residues, important for the interpretation of fragments resulting from cyanogen bromide cleavage. As for the tryptophan residues, the results from different laboratories are not consistent with the number of six, as will be discussed later, but the tryptophan sequences are interesting, because of this low number. Other features of the molecule are of course of interest, the amino and carboxyl ends, the serine bearing the phosphate group and, of course, the active centre.

Allow me now to summarize what is known about all these points. The active site of pepsin will be described certainly in a more competent way by the next speaker. I will quote only the three results, published this year on the sequences around that aspartic acid residue directly related to the active site:

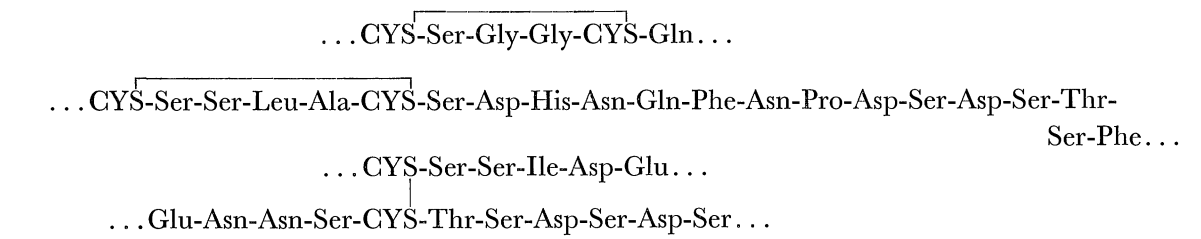
| | |
|---|---|
| Ile-Val-ASP-Thr-Gly-Thr-Ser | Bayliss & Knowles (1968); Bayliss *et al.* (1969) |
| Val-ASP | Stepanov & Vaganova (1968) |
| Ile-Val-ASP-Thr | Fry *et al.* (1968) |

The first peptide was obtained after inactivation of pepsin by *N*-diazoacetyl-L-phenylalanyl-methylester, the second by *N*-diazoacetyl-*N'*-2,4-dinitrophenyl-ethylenediamine and the last by 1-diazo-4-phenyl-butan-2-one. Evidence was presented that this active grouping is not located within the first third of the polypeptide chain of pepsin, because a fragment containing the label and lacking histidine has been isolated (Stepanov & Vaganova 1968).

What do we know about the serine residue labelled with the phosphate group? The phosphate bears probably no direct relation to the activity. In human pepsin phosphate is lacking (Solovjeva & Ginodman) and the enzyme is still active. Flavin (1954) isolated from hog pepsin a tripeptide with bound phosphate. This sequence was later extended by Stepanov, Vachitova, Jegorov & Avajeva (1965) to a heptapeptide structure and recently to a hendecapeptide (Stepanov 1968):

| | |
|---|---|
| Thr-SER-Glu | Flavin (1954) |
| Glu-Ala-Thr-SER-Glu-Glu-Leu | Stepanov *et al.* (1965) |
| Glu-Ala-Thr-SER-Glu-Glu-Leu (Ile,Thr,Ser) Tyr | Stepanov *et al.* (1968) |

Let us summarize now our information on the sulphur containing sequences. According to amino acid analysis, there are three disulphide bridges in pepsin. The linkage of these bridges was determined independently by Tang & Hartley (1967) and by Keil, Morávek & Šorm (1967); the results are in agreement. Below are the results of Tang & Hartley (1969), because their sequences are more complete:




A very characteristic feature emerges from these results: at least two of the three disulphide bridges in pepsin form very short loops and therefore do not contribute to the tertiary structure of linearly distant parts of the polypeptide chain.

As regards sequences around the methionine residues, there are no published data except three dipeptides mentioned by Stepanov, Katrukha & Ostoslavskaja (1966): Met-Val, Met-Asp (twice) and Met-Gly (?). But it has been a public secret that all these sequences are well known but concealed in the log-book of Dr Hartley in Cambridge. With his authorization I included the results in this survey. The four following sequences (Tang & Hartley 1969) were obtained by using diagonal electrophoresis:

Asp-Ser-Ile-Thr-MET (Asp,Glu,Gly) (Ala,Tyr)
Ala-Ser-Glu-Asn-Ser-Asp-Gly-Glu-MET-Ile (Val,Tyr)
Glx-Gly-Asx-Ser (Thr, Ser,Val) Glu-Gly-Asp-MET
Tyr-Gly-Thr-Gly-Ser-MET-Asp-Val-Pro-Thr-Ser

They are of great value in connexion with the cyanogen bromide cleavage of the pepsin molecule because they represent the overlapping structures.

Of the sequences containing tryptophan, four were established this year by Dopheide & Jones (1968) from the Rockefeller University by substitution of the reduced carboxymethylated pepsin with 2-hydroxy-5-nitro-benzyl bromide and by degradation using chymotrypsin and thermolysin:

I Val-Phe-Asp-Asn-Leu-TRP-Asp-Gln-Gly
II Leu-TRP-Val-Pro-Ser
III Val-Glu-Gly (TRP,Gln)
VI Leu-Asn-TRP-Val-Pro

One was obtained by Kostka, Morávek & Keil (1969) from a 49 residue fragment representing the C-terminal portion of pepsin:

V ...Ser-Ser-Gly-Glu-Leu-TRP-Ile (Asp,Thr,Ser,Ser,Glu,Pro, Gly,Val,Leu,Tyr,Phe) Ile-Leu-Gly...

Two were assigned by Stepanov (1968) to an analogous part of the molecule:

VI ...Ser (Ser,Gly,Glu) Leu-TRP-Thr-Ile (Asp,Ser,Ser,Glu,Pro, Gly,Val,Leu) Ala-TRP-Ile-Leu-Gly...

That makes altogether six sequences. According to Dopheide & Jones (1968) only two of the tryptophan residues (I and IV) react in native pepsin, and the modified enzyme retains 70% of its activity. After denaturation in alkaline medium a third residue reacts, whereas the fourth is accessible only after complete unfolding of the molecule by reduction of the disulphide bridges. You will remember that two of the three disulphide bridges form very short loops, so the rearrangement of the molecule uncovering the fourth tryptophan may be attributed to the splitting of the third disulphide bond.

Now, how many tryptophans are there in fact in pepsin? Earlier spectrophotometric analyses have shown a tryptophan content of six residues (Blumenfeld & Perlmann 1959; Arnon & Perlmann 1963). Dopheide & Jones (1968) obtained the same result when using a spectrophotometric method and concluded that spectral measurements did not work in the special case of pepsinogen and pepsin. Titration with *N*-bromosuccinimide also gave 5–6 tryptophan residues (Lokshina & Orekhovich 1964; Green & Witkop 1964), but when these experiments were repeated by the Rockefeller group on commercial and pure pepsin the results were 5 and 3 residues/molecule, respectively. This shows clearly that the differences may either be attributed to the inhomogeneity of commercial pepsin, or that the structure of the intact enzyme influences the analytical results. Now how to interpret the results shown above? Because the sequences V and VI are from the same C-terminal fragment, one of them must be wrong. In our laboratory using the reaction of the isolated fragment with 2-nitrophenylsulphenyl chloride the presence of only one tryptophan residue was found. The sequence presented here (V) is based on two pieces of evidence: a hexapeptide of unambiguous composition with C-terminal tryptophan was isolated by Kostka from the 49 residue fragment, and the sequence up to the isoleucine residue was established by Kluh both in the automatic sequenator and also by the stepwise manual technique. In both cases the identification of isoleucine or leucine was somewhat marginal, but the presence of valine could be eliminated. This fact speaks against the possibility of placing sequence II in the C-terminal fragment. The isolation of a labelled tryptophan peptide arising from this sequence is under way in our laboratory, and we hope that it will throw more light on this discrepancy.

How far do we know the amino terminal sequence of pepsin? The end group was determined as isoleucine by Sanger (1945) 23 years ago. Williamson & Passmann (1956) proposed a terminal sequence of six amino acid residues including histidine which must be considered incorrect in view of recent data. Last year a large fragment was isolated from a cyanogen bromide hydrolysate of S-carboxymethyl-pepsin in the laboratory of Stepanov, and the dipeptide sequence of its amino end was determined (Vachitova, Ostoslavskaja, Krivitzov & Stepanov 1967). A tetrapeptide sequence was identified in an analogous fragment in our laboratory (Trufanov, Kostka, Keil & Šorm 1969), and recently Stepanov (1968) reported a pentapeptide sequence which obviously comes from the same N-terminal portion of the molecule:

| | |
|---|---|
| $H_2N$-Ile | Sanger (1945) |
| Leu-Gly-Asp-Asp-His-Glu- | Williamson & Passmann (1956) |
| Ile-Gly(150–160 res) | Vachitova *et al.* (1967) |
| Ile-Gly-Asp-Glu(156 res) | Trufanov *et al.* (1969) |
| Ile-Gly-Asp-Glu-Pro(—) | Stepanov (1968) |

The C-terminal structure is much better known. It has a very favourable composition, with two arginine and one lysine residue, which gives the opportunity of obtaining small peptides

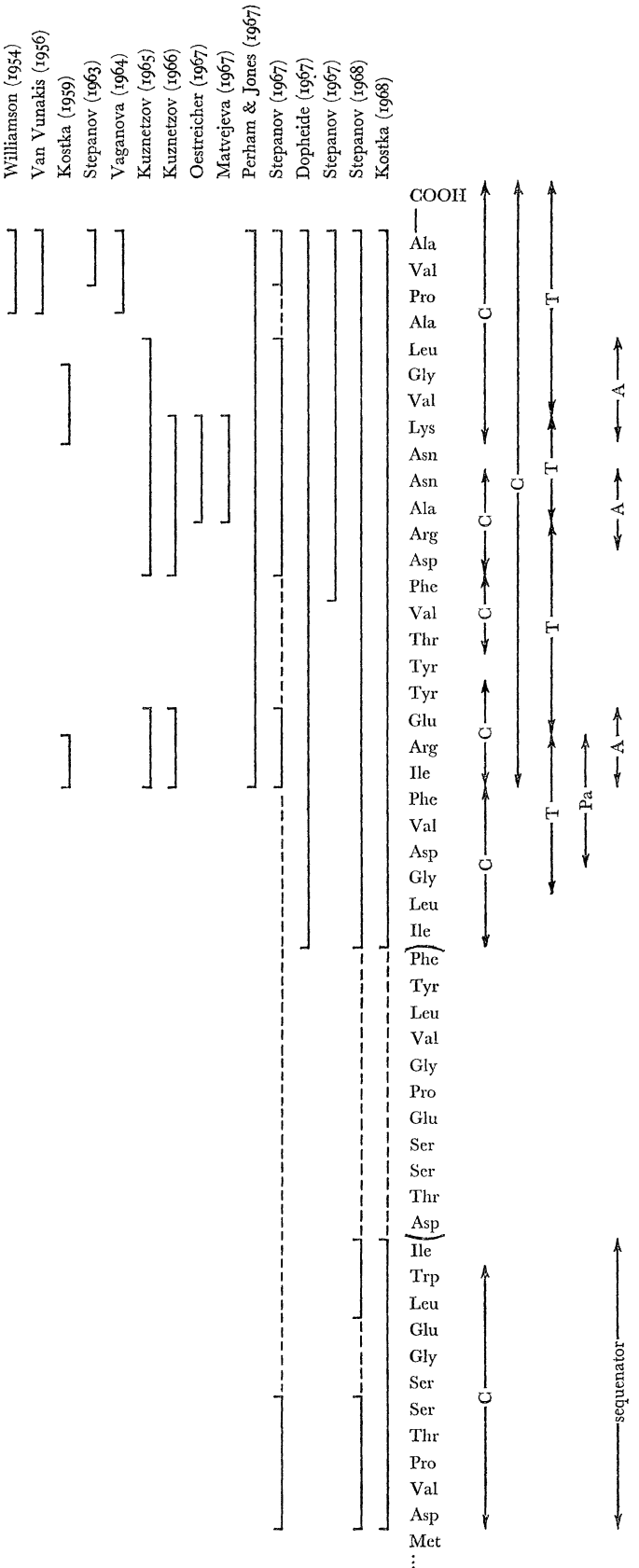



FIGURE 1. Fragments derived from the C-terminal part of pepsin as reported by different laboratories (upper part) and sequence studies on this fragment by Kostka *et al.* (37) (lower part). Arrows denote sequences established by the Edman technique or carboxypeptidase degradation in peptides isolated from chymotrypsin (C), trypsin (T), papain (Pa) or pepsin (P) digests.

from the tryptic digest of pepsin. The history of this part of the molecule is full of results from six different laboratories (figure 1).

The first information obtained from carboxypeptidase digestion (Williamson & Passmann 1954; Van Vunakis & Herriott 1956) had to be revised in view of the recent results. Kostka, Keil & Šorm (1959) described two basic peptides and Stepanov & Greil (1963) and Vaganova, Levin & Stepanov (1964) corrected the C-terminal sequence. Then began a hunt for peptides containing basic amino acids. From a chymotryptic and peptic digest of pepsin and of reduced and carboxymethylated pepsin the Russian authors isolated and reported two fragments (Kuznecov & Stepanov 1965, 1966); from a tryptic digest two other peptides (Matvejeva & Stepanov 1967). Among the basic peptides obtained from a tryptic hydrolysate of pepsinogen by Oestreicher & Perlmann (1967) one tetrapeptide fragment can also be placed in the carboxyl end of the molecule.

Much more complete information resulted from the data published during the last two years. Perham & Jones (1967), using the technique of diagonal electrophoresis for the isolation of peptides resulting from tryptic cleavage of trifluoracetylated pepsin were able to determine 20 amino acid residues. Stepanov, Ostoslavskaya, Krivitzov, Muratova & Levin (1967*a*) announced preliminary information on the study of a fragment from a cyanogen bromide hydrolysate. A large sequence of 27 amino acid residues was determined by Dopheide, Moore & Stein (1967) from the Rockefeller University. They started with pure pepsin freshly prepared from pepsinogen. Tryptic and chymotryptic digests of this pepsin and of its reduced and aminoethylated and trifluoracetylated derivatives led to the final structure. Another check of the correctness of a part of this sequence comes from the data of Stepanov, Matvejeva & Krivitzov (1967*b*) on the tryptic hydrolysate of carboxymethylated pepsin.

In 1968 new details were given by two laboratories on one of the large fragments resulting from cyanogen bromide cleavage either of *S*-sulphopepsin (Kostka *et al.* 1968) or of reduced and carboxymethylated pepsin (Stepanov 1968). Our results differ from those of Stepanov in some details. First, there are two tryptophans in the sequence given by Stepanov, and secondly there are different neighbours of the first tryptophan. On the lower part of figure 1 more details are given on the kind of work with this fragment undertaken by Kostka in our laboratory (Trufanov *et al.* 1969).

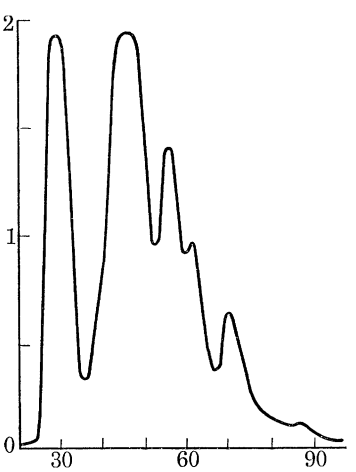



FIGURE 2. Fractionation of the cyanogen bromide hydrolysate of pepsin on Sephadex G-100 (8.0 mol $l^{-1}$ of urea; 0.3 mol $l^{-1}$ of ammonium acetate, pH 6.0).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Several times I mentioned here large fragments of pepsin resulting from cyanogen bromide cleavage. This method of fragmentation of carboxymethylated pepsin was first announced by Ostoslavskaya, Krivitzov, Katrukha, Muratova, Levin & Stepanov in 1966 and was independently used in our laboratory with *S*-sulpho-pepsin (Kostka *et al.* 1968; Trufanov *et al.* 1969). Both derivatives give essentially the same pattern when the hydrolysate is fractionated on Sephadex G-200 or G-100 in solutions containing urea (figure 2).

The first peak, although its elution volume is very low, contains the relatively short 49 residue fragment from the C-terminus, whose sequence was given in figure 1. The next two peaks give after rechromatography large fragments containing about 160 amino acid residues and representing the N-terminal half of the molecule. Up to now we are not able to pick up a defined fraction of the remaining 110 residues filling the gap between this fragment and the C-terminal sequence. The large fragment contains one of the three disulphides:

Ile-Gly-Asp-Glu(Asp$_{14}$,Gly$_{11}$,Thr$_{10}$,Leu$_{10}$,Ser$_9$,Glu$_8$,Val$_7$,Ile$_7$,Tyr$_7$,Phe$_7$,Pro$_5$,Ala$_3$,Tyr$_2$)
CYS(Ser,Ser,Leu,Ala,CYS,Ser,Asp,His,Ser,Asp,Glu,Phe,Asp,Ser$_2$,Asp,Thr,Pro,Phe,Asp$_2$,
Ser$_7$,Gly$_6$,Glu$_4$,Leu$_4$,Ala$_3$,Ile$_2$,Pro$_2$,Tyr$_2$,Thr,Val)-MET

After its reduction and substitution with ethylenimine and subsequent splitting with trypsin, a fragment of 105 residues with C-terminal aminoethyl-cysteine can be obtained in a pure state with a good analysis (Trufanov *et al.* 1969). It is devoid of histidine and its N-terminal sequence is identical with that of pepsin. This means that it respresents the first third of the whole molecule.

The work on the structure of pepsin continues in several laboratories and as a last illustration allow me to present some brand new unpublished data obtained in the laboratory of Stepanov, which professor Andreeva brought to this meeting and asked me just before my lecture to include into my survey. I am not familiar with the details and have not seen the data before; therefore those results which were not already included in my preceding review are shown below:

Tyr-Ala-Pro-Phe
Asp-Gly-Ile-Gly-Leu-Leu
Leu-Gly-Gly-Ile-Asp-Ser-Ser-Tyr-Tyr
Leu-Tyr
Ser-Val-Tyr
Val-Ile...Ser-Pro-Ser-Ala-Tyr...Glu-Gly-Met
Asp-Gly-Glu-Thr-Ile

Here the survey of the known and published sequences of pepsin should end. But it would be incomplete because an inherent part of the chemistry of pepsin is its precursor, pepsinogen. During the activation of pepsinogen a family of fragments is split off its N-terminal region and accounts for 41 residues. In the scheme given below two amino acid sequences proposed for this region by two different laboratories are presented. They share only one tetrapeptide structure in common:

Leu-Val-Leu-Glu-Pro-Ala-Glu-Phe-Ser-Leu-Lys-Asp-Gly-Lys-Val-(Asp,Pro)-Leu
(Lokshina & Orechovich 1960)

Leu-Val-Lys-Val-Pro-Leu-Val-Arg-Lys-Lys-Ser-Leu-Arg-Gln-Asn-Leu-Ile-Lys-Asp-Gly-Lys-Leu-Lys-Asp-Phe-Leu-Lys-Thr-His-Lys-His-Asn-Pro-Ala-Ser-Lys-Tyr-Phe-Pro-Ala-Glu (Koehn & Perlmann 1968)


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The sequence reported by Koehn & Perlmann differs only slightly from the amino acid composition which may be expected on the basis of difference in amino acid composition of pepsinogen and pepsin.

$H_2N$-Ile-Gly-Asp-Glu-Pro [/ $Asx_9$,$Gly_4$,$Thr_7$,$Leu_3$,$Ser_2$,$Glx_2$,$Val_5$,$Ile_4$,Tyr,$Phe_4$,$Pro_2$,Ala/Glu-Ala-Thr-Ser(P)-Glu-Glu-Leu-Ser(Thr,Ile)Tyr / Tyr-Ala-Pro-Phe / Asp-Gly-Ile-Gly-Leu-Leu / Val-Phe-Asp-Asn-Leu-Trp-Asp-Gln-Gly- / Leu-Gly-Gly-Ile-Asp-Ser-Ser-Tyr-Tyr / Gly-Leu-Ser-Glu-Thr-Glu-Pro-Gly-Ser-Phe / Leu-Tyr / ]Ser-Val-Tyr / -Cys-Ser-Ser-Leu-Ala-Cys-Ser-Asp-His-Asn-Gln-Phe-Asn-Pro-Asp-Ser-Asp-Ser-Thr-Ser-Phe-($Ser_6$,$Glx_4$,$Pro_2$,$Gly_6$,$Leu_4$,$Ala_3$,$Tyr_2$,Ile,Val)-Asp-Ser-Ile-Thr-Met-(Asp,Gly,Glu)(Ala,Tyr)-[/ Glu-Asn-Asn-Ser-Cys-Thr-Ser-Asp-Ser-Asp-Ser / Cys-Ser-Ser-Ile-Asp-Glu / Leu-Trp-Val-Pro-Ser / Asp-Gly-Glu-Thr-Ile / Ser-Pro-Ser-Ala-Tyr / Val-Glu-Gly-(Trp,Gln) / Leu-Asn-Trp-Val-Pro / Ala-Ser-Glu-Asn-Ser-Asp-Gly-Glu-Met-Ile-(Val,Tyr) / Glx-Gly-Asx-Ser-(Thr,Ser,Val)-Glu-Gly-Asp-Met / Cys-Ser-Gly-Gly-Cys-Gln / Ile-Val-Asp*-Thr-Gly-Thr-Ser / $Leu_7$,$Ile_6$,$Ala_3$,$Thr_4$,$Pro_2$,$Phe_2$, Gly,Ser,Asp /]-Tyr-Gly-Thr-Gly-Ser-Met-Asp-Val-Pro-Thr-Ser-Ser-Gly-Glu-Leu-Trp-Ile(Asp,Thr,$Ser_2$,Glu,Pro,Gly,Val,Leu,Tyr,Phe)-Ile-Leu-Gly-Asp-Val-Phe-Ile-Arg-Gln-Tyr-Tyr-Thr-Val-Phe-Asp-Arg-Ala-Asn-Asn-Lys-Val-Gly-Leu-Ala-Pro-Val-Ala-COOH

FIGURE 3. Partial structure of pepsin. Determined fragments, whose relative position is unknown, are separated by /. Aspartic acid residue contributing to the active site is marked by *.

And now comes the last survey of this talk (figure 3), summarizing the known structure of pepsin. The number of peptide sequences determined unambiguously is about two-thirds of the molecule and the N-terminal half of the molecule is in the form of a well defined fragment whose further fragmentation is in progress. The sequence of the last 55 amino acid residues at the carboxyl end is practically solved, and there remains a region of about 100 residues where we cannot determine the relative position of the fragments. But let us hope for the best.

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