

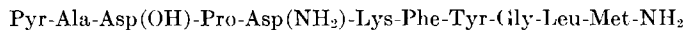
Isolation and Amino Acid Sequence of Physalaemin, the Main Active Polypeptide of the Skin of *Physalaemus fuscumaculatus*¹

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Received July 1, 1964

Crude methanol extracts of fresh and dried skins of *Physalaemus fuscumaculatus*, a South American amphibian, were subjected successively to chromatography on alkaline alumina and to countercurrent distribution. The main active constituent of these extracts, the polypeptide *physalaemin*, was thus obtained in a pure form. Total and partial acid hydrolysis, fission with trypsin and chymotrypsin, and end-group determination experiments demonstrated that *physalaemin* is an endecapeptide having the following amino acid composition and sequence:



Some anomalies in the trypsin and chymotrypsin fission of *physalaemin* are discussed, and the strict chemical resemblance between *physalaemin* and *eledoisin* is pointed out.

INTRODUCTION

Methanol extracts of dry or wet skin of *Physalaemus fuscumaculatus*, a South American amphibian, possess a powerful hypotensive action in some experimental animals, and display a potent stimulant action on several extravascular smooth muscles (1).

The present communication describes the experiments which have led to the isolation of the active principle and to its identification with an endecapeptide having a structure similar to that of *eledoisin* (2, 3). A preliminary report on this topic has already appeared (4).

MATERIALS AND METHODS

The *Physalaemus* material considered in this study was as follows:

(a) 323 adult specimens captured near Tucuman (Argentina) in January-February, 1963. The dried

skins weighed all together 32.8 gm, and the average weight of a skin was 0.10 gm. The *physalaemin* content was 650 μg per gram dry skin.

(b) 471 adult specimens captured at the same time as the preceding ones, at the same place. The fresh skins weighed all together 206 gm, and the average weight of a skin was 0.43 gm. The *physalaemin* content was 150 μg per gram fresh skin.

(c) 275 adult specimens captured in part near Tucuman and in part near Cordoba in December, 1963. The total weight of the dried skins was 43 gm, and the average weight of a single skin was 0.16 gm. The *physalaemin* content was 700 μg per gram dry skin.

Having found that one dried skin contained exactly the same amount of active principle as one fresh skin, methanol extracts of lots a and b were combined to form the *standard extract Physalaemus 1963*, which was used in the present investigation. The concentration of this extract was always expressed in terms of fresh skin.

Extracts of fresh skins were prepared in Argentina by extracting twice with 5 parts (w/v) of methanol the skins removed from the animals immediately after their sacrifice. The skins selected to be dried were carefully spread out and

¹ This work was supported in part by a grant from the Consiglio Nazionale delle Ricerche, Roma.

were dried in the shade. Immediately after their arrival in Italy, by air mail, they were minced with scissors and then immersed in 10–15 parts of 80% methanol. The liquid was decanted after a week, and the skins were extracted for another week with 15 parts of the solvent. The methanol extracts, yellow in color, were combined and filtered. When kept in dark bottles and refrigerated, they may be stored for months and even years without appreciable loss of activity.

The two test systems most frequently used in the bioassay of physalaemin, owing to their high sensitivity and the excellent dose-response relationship, were the dog blood pressure and the isolated rabbit large intestine. The threshold dose of pure natural physalaemin capable of lowering the blood pressure of the anesthetized dog by intravenous injection is of the order of 1 ng per kilogram; the threshold dose capable of stimulating the isolated rabbit large intestine is 0.5–1 ng per milliliter. Other preparations and test systems which were used in the bioassay of physalaemin, especially in parallel assays, were the rabbit blood pressure, the chicken blood pressure, the isolated guinea-pig ileum, and isolated intestinal and/or uterine smooth muscles from rabbits, rats, dogs, and pigeons (5, 6).

The techniques and materials used for the purification and the degradative steps of physalaemin were essentially the same as those previously adopted for similar experiments with eledoisin (3), except that in all cases the peptide fragments obtained upon partial enzymic or chemical cleavage were separated by high voltage electrophoresis on thick Macherey No. 214 paper (from Nagel and Co., Düren) previously exhaustively washed with a 50% ethanol-water mixture. An apparatus from L. Hormuth, Inh. W. E. Vetter (Heidelberg), according to Wieland and Pfeleiderer, was used for all the electrophoresis experiments.

ISOLATION OF PHYSALAEAMIN

Adsorption on alkaline alumina. In a typical experiment, 675 ml of the *Physalaemus* 1963 standard extract, corresponding to 75 gm fresh skin, was evaporated, under reduced pressure and at 45–50°C, to 50–60 ml, and the remaining aqueous liquid was extracted repeatedly with petroleum ether in order to remove fats. The distillation was then continued until the residue was of syrupy consistence. The residue (18 mg per gram fresh tissue) was taken up in a warm water bath by stirring in 100 ml of 95% ethanol. The abundant brownish precipitate found after storage overnight in the refrigerator was discarded, and the liquid was passed through a column of 140 gm (21 × 3.3

cm) of alkaline alumina. Development of the chromatogram was effected by successive addition of 200–300 ml each of 95, 90, 80, 70, 60, 50, 40, and 30% ethanol, and 200 ml of distilled water. Fractions of 100 ml were collected. Bioassay showed that 60–70% of the physalaemin activity appeared in the 60% ethanol eluates, more precisely in eluates 60B and 60C (Fig. 1).

Two minor polypeptides, active on the rat colon and the rat estrous-uterus but with no or negligible activity on the rabbit large intestine, were eluted by 80 and 70% ethanol, respectively. Their pharmacological study is in progress.

Paper chromatography and electrophoresis soon demonstrated that the spot containing the physalaemin activity gave sharp positive reactions not only to ninhydrin and chlorine, but also to the iodoplatinate reagent of sulfur amino acids (7) and the α -nitroso- β -naphthol reagent of tyrosine (8).

The only biogenic amine occurring in minute amounts (2.5 μ g per gram fresh tissue) in the *Physalaemus* skin was leptodactyline. It was eluted by 95% ethanol.

Countercurrent distribution. On the whole an amount of material equiactive to roughly 150 gm fresh tissue was obtained from three alumina columns. It had a dry weight residue of approximately 120 mg, i.e., 0.8 mg per gram fresh tissue.

For further purification, aliquots of this material containing the activity of about 50 gm fresh tissue were evaporated to dryness under reduced pressure and then submitted to a double countercurrent distribution of 40 transfers in a hand-operated train with 25 ml of lower and upper phases capacity. The systems used were respectively *n*-butanol-ethanol-acetic acid-water (80:16:16:128) with a pH of 2 in the lower phase for the first, and *n*-butanol-pyridine-water-0.1 *N* ammonia (68:41:150:2) with a pH of 9 in the lower phase, for the second separation. The active material showed distribution coefficients of 1.1 in the first, and 1.3 in the second system and was recovered at the end of the second run with a yield of activity of 70–75%. An amount of this purified product equiactive to 1 gm fresh tissue had a dry weight residue averaging from 80 to 100 μ g and contained 65–70 μ g of physalaemin as shown by quantitative amino acid analysis.

On high-voltage paper electrophoresis, the active spot migrated toward the cathode at acidic pH, its relative position being $E_{1.9} = 0.43$ (Glu (7)), and showed no mobility at pH 5.8, indicating an almost neutral isoelectric point. On ascending paper chromatograms it had an R_f value of 0.41 in the system *n*-butanol-acetic acid-water (40:10:50), and of 0.38 in the system *sec*-butanol-1.5 *N* ammonia (75:25). A single active spot com-

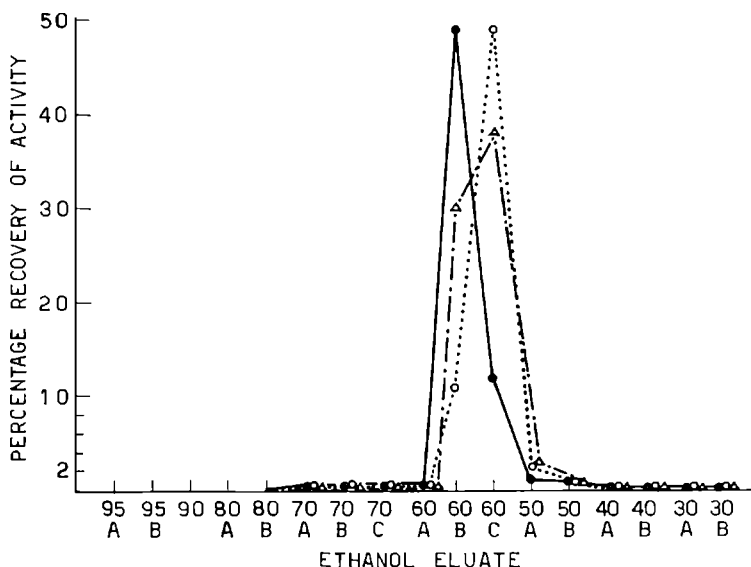


FIG. 1. Elution of physalaemin from three different alkaline alumina columns with descending concentrations of ethanol. In all three experiments the peak of activity, as assessed on the rabbit large intestine, appeared in the 60% ethanol eluates.

pletely free of contaminants was obtained in all the systems tested.

DETERMINATION OF AMINO ACID COMPOSITION

Portions of the pure product having the activity of 2-3 gm fresh skin were hydrolyzed with 6 *N* HCl at 105°C in tubes sealed under nitrogen for 18-20 hours, and the reaction mixtures were subjected to bidimensional chromatography run with high-voltage electrophoresis in the first dimension and ascending chromatography in the *n*-butanol-acetic acid-water system in the second dimension. The amino acids found in the hydrolyzate were glutamic and aspartic acids, alanine, proline, lysine, phenylalanine, tyrosine, glycine, leucine, and methionine. Owing to the scarcity of material, only a paper method could be used for quantitative amino acid analysis. However, sufficiently accurate results were obtained by repeating the experiments several times in comparison with standard amounts of pure amino acids subjected to parallel experiments of bidimensional chromatography, elution of the spots and determination of the color intensity. On the other hand, the stepwise enzymic and chemical degradation described in the next section fully confirmed the amino acid composition of the total acid hydrolyzate.

Amino acid estimation was carried out with the method described by Blackburn and Lee (9) for all the amino acid residues except proline, which

was estimated after reaction with isatine upon direct comparison with spots produced by different amounts of standard proline. The results showed that nine out of ten residues were present in a 1:1 mole ratio; only the aspartyl residue was present in a 2:1 mole ratio with respect to the other amino acids.

DETERMINATION OF AMINO ACID SEQUENCE

Physalaemin, like eledoisin (2, 3) is resistant to enzymic and chemical attack at both the C and N terminus. Although a number of attempts were made, it was not possible to split the N or C terminal residues with either leucinamino-peptidase or carboxypeptidase digestion; correspondingly no loss of biological activity was observed in the active material exposed to the action of the enzymes for periods of time as long as 15 hours. Also the chemical techniques with fluoro-2,4-dinitrobenzene and phenylisothiocyanate failed in degrading the molecule at the N side.

The amino acid sequence of physalaemin was elucidated by enzymic cleavage with chymotrypsin and trypsin followed by the analysis of the resulting fragments. Indicative results were also obtained with partial acid hydrolysis of the total molecule and of the N-terminal tryptic fragment. The status of the ionic groups was deduced from the migration speed of both the intact molecule and the peptide fragments, subjected to high-voltage electrophoresis in different systems.

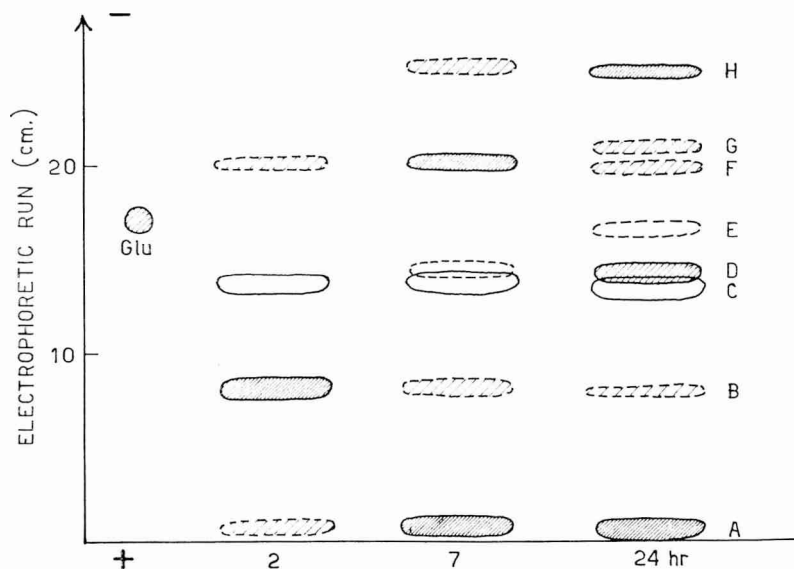


FIG. 2. High-voltage electrophoresis at pH 1.9 of a chymotryptic digest of physalaemin. Voltage gradient, 45 V per centimeter. Electrophoretic pattern after 2, 7, and 24 hours of digestion. Band A (origin) stained only with chlorine, Pyr-Ala-Asp(OH)-Pro-Asp(NH₂)-OH; band B ($E_{1.9} = 0.47$ (Glu), Pyr-Ala-Asp(OH)-Pro-Asp(NH₂)-Lys-Phe-Tyr-OH; band C ($E_{1.9} = 0.83$ (Glu), H-Gly-Leu-Met-NH₂; band D ($E_{1.9} = 0.85$ (Glu), H-Tyr-OH; band E ($E_{1.9} = 0.95$ (Glu), H-Gly-Leu-OH; band F ($E_{1.9} = 1.2$ (Glu), H-Lys-Phe-Tyr-OH; band G ($E_{1.9} = 1.3$ (Glu), H-Met-NH₂; band H ($E_{1.9} = 1.9$ (Glu), H-Lys-Phe-OH.

to check the results three different batches of the enzyme thrice crystallized were tested, two of them from Fluka A. G., Buchs, Switzerland and one from Princeton Laboratories, Princeton, New Jersey. All of them behaved in exactly the same way with physalaemin and with the synthetic peptide, H-Phe-Tyr-Gly-Leu-Met-NH₂, but they showed no chymotryptic action on other substrates specific for chymotrypsin (e.g., bradykinin).

Finally it was seen that both the specific and the aspecific splitting by trypsin of the -Lys-Phe- and the -Tyr-Gly- bonds could be inhibited by the specific soybean trypsin inhibitor (100 μ g physalaemin in 0.2 ml of 0.1 M ammonium acetate at pH 8.2 + 50 μ g trypsin + 100 μ g soybean inhibitor, 4 hours at 37°C).

Incubation of T₁ with carboxypeptidase-B caused the rapid release of lysine and the production of a fragment on which carboxypeptidase-B and carboxypeptidase-A had no further action. It was clearly the same fragment C₃ produced by chymotrypsin which was unreactive to ninhydrin and showed the electrophoretic mobility of a negatively charged peptide.

With the above results the sequence -Lys-Phe-

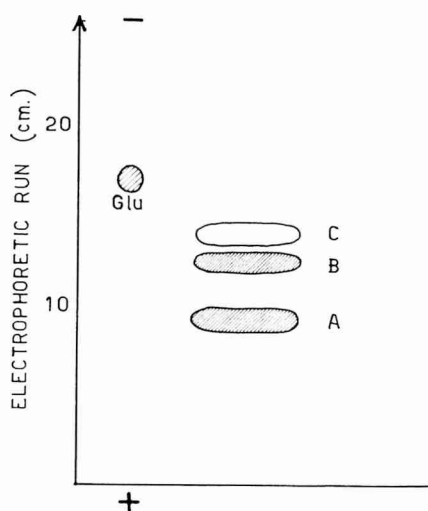
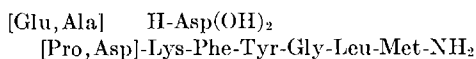


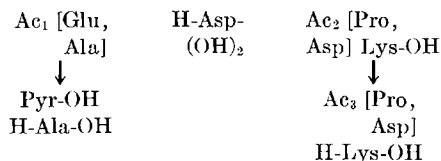
FIG. 3. High-voltage electrophoresis at pH 1.9 of a tryptic digest of physalaemin. Voltage gradient, 45 V per centimeter. Band A ($E_{1.9} = 0.56$ (Glu), Pyr-Ala-Asp(OH)-Pro-Asp(NH₂)-Lys-OH; band B ($E_{1.9} = 0.74$ (Glu), H-Phe-Tyr-OH; band C ($E_{1.9} = 0.83$ (Glu), H-Gly-Leu-Met-NH₂.

Tyr-Gly-Leu-Met-NH₂ could be assigned to the C-terminal hexapeptide. The sequence of the N-terminal pentapeptide [Glu,Ala,2 Asp,Pro] was elucidated on the basis of the data obtained with partial acid hydrolysis experiments.

Partial acid hydrolysis. The hydrolyses were carried out with 0.2 μmole of physalaemin or with an amount of fragment T₁ produced by tryptic hydrolysis of 0.4 μmole of physalaemin. The samples were dissolved in 0.3 ml of 0.5 M acetic acid and were allowed to stand in sealed tubes at 100°C for 15 and 40 hours. It was soon evident that physalaemin was split in essentially the following way with release of free aspartic acid



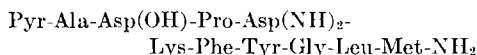
while the splitting products of T₁ were consecutively



Ac₁ was negative to ninhydrin and migrated toward the anode when submitted to electrophoresis at pH 5.8. Upon further action of dilute acid it gave alanine and a spot negative to ninhydrin which was readily identified as pyroglutamic acid.

Ac₂ showed the electrophoretic mobility of a positively charged peptide in acid and neutral medium, while the mobility of Ac₃ indicated that it was positively charged at pH 1.9 and neutral at pH 5.8. Therefore it could be concluded that they contained an asparaginyl residue. Finally both Ac₂ and Ac₃ gave positive reactions with isatine, indicating an N-terminal position of proline.

The above results were unequivocally conclusive for the assignment of the N-terminal structure, and consequently the complete sequence of physalaemin was formulated as follows:



IDENTITY OF PURE NATURAL PHYSALAEMIN WITH THE ACTIVE PEPTIDE PRESENT IN CRUDE SKIN EXTRACTS AND WITH SYNTHETIC PHYSALAEMIN

It is known that an N-terminal glutamine can be converted into a pyroglutamic residue during the isolation procedures (16-18). Thus the problem arose, exactly as in the case of eledoisin, of whether the endecapeptide obtained following

the purification procedures described herein possessed the same N-terminal structure of the peptide present in the crude methanol extracts of the skin. We attempted to solve this problem by paper electrophoretic experiments and by parallel biological assays of different physalaemin preparations in numerous test systems.

Amounts of crude methanol extracts corresponding to 1 mg fresh skin were subjected to high-voltage paper electrophoresis in parallel with 1 μg of pure natural physalaemin and 1 μg of pure synthetic physalaemin. It was found that the relative position on pherograms of the active spots was always the same ($E_{1,9} = 0.43$ Glu). Moreover, electrophoresis of mixtures of crude methanol extract and pure physalaemin always gave a unique active spot.

The following four physalaemin preparations were subjected to parallel bioassay: (a) the crude standard *Physalaemus* extract 1963; (b) a 60% ethanol eluate from an alumina column; (c) pure natural physalaemin; and (d) synthetic physalaemin purified by two successive countercurrent distributions.

The three first preparations were suitably diluted so that they possessed exactly the same hypotensive action in the dog. Synthetic physalaemin was always used at the same concentration as natural physalaemin. These preparations were then assayed in parallel on rabbit blood pressure, chicken blood pressure, rabbit large intestine, guinea-pig ileum, and pigeon duodenum. The activity of the *Physalaemus* standard extract was always considered equal to 100, and the activity of the other preparations was expressed in per cent.

Table I clearly shows that doses of the three preparations of natural physalaemin which are equiactive on the dog blood pressure are also equiactive in all other test systems, i.e., that the pure natural polypeptide is indistinguishable, by bioassay also, from the polypeptide present in crude skin extracts. It further shows that synthetic physalaemin is identical with natural physalaemin.

In connection with the problem discussed in this section, it seems pertinent briefly to describe some recent experiments on eledoisin.

Crude methanol extracts of posterior salivary glands of *Eledone* (10) corresponding to 1-2 mg fresh tissue were subjected to high-voltage paper electrophoresis in parallel with pure natural eledoisin, pure synthetic eledoisin, and synthetic glutamine eledoisin, Glu(NH₂)-Pro-Ser-Lys-Asp(OH)-Ala-Phe-Ile-Gly-Leu-Met-NH₂. Whereas the position of the active polypeptide spots given by the three first preparations was exactly the same ($E_{1,9} = 0.43$ Glu), glutamine

TABLE I
RESULT OF PARALLEL BIOASSAY OF FOUR PHYSALAEAMIN PREPARATIONS ON SEVEN TEST OBJECTS^a

Test object	Relative activity (%)			
	<i>Physalaemus</i> 1963 standard extract	60% ethanol eluate	Pure natural physalaemin	Synthetic physalaemin
Dog blood pressure	100	100	100	100
Rabbit blood pressure	100	105-115	100-110	95-105
Chicken blood pressure	100	90	110	100
Rabbit large intestine	100	105	100-105	95-105
Guinea-pig ileum	100	105	95-100	95-105
Dog large intestine	100	95	95	—
Pigeon duodenum	100	95	—	—

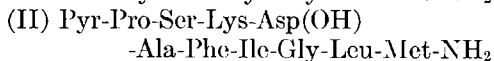
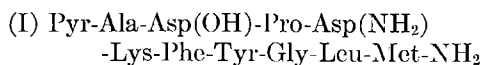
^a The activity of the *Physalaemus* 1963 standard extract was always considered equal to 100; that of the other three physalaemin preparations was expressed in per cent.

eledoisin, as expected, migrated toward the cathode faster than eledoisin ($E_{1.9} = 0.76$ Glu). On the other hand, solutions of glutamine eledoisin in ethanol or methanol have been stored for months without any appreciable transformation of the N-terminal glutamine into a pyroglutamic residue. The conclusion from these two sets of experiments is that eledoisin is the very polypeptide present in the fresh salivary tissue of *Eledone*.

Although we have as yet not had the opportunity of examining glutamine physalaemin, it seems that the above conclusions for eledoisin may be analogically extended to physalaemin.

DISCUSSION

The most interesting fact which has emerged from the present study is that of the strict chemical resemblance existing between physalaemin (I) and eledoisin (II)



This resemblance satisfactorily explains the striking similarity in the biological effects of the two polypeptides, which has allowed us to ascribe to physalaemin the attribute "eledoisin-like" long before the elucidation of its chemical structure was accomplished (1).

The two above formulations show that the N-terminal amino acid residue is the same for physalaemin and eledoisin and, what is still more important, at least from the point of view of biological activity, the

C-terminal tripeptide is exactly the same also. Yet eledoisin is found in the salivary glands of an invertebrate, and physalaemin in the skin of a vertebrate.

Something similar has been observed in the case of the bradykinins. The amphibian skin contains a number of bradykinin-like polypeptides, but one of them, the bradykinin of *Rana temporaria* skin, is identical to the bradykinin originating in human blood by the action of proteolytic enzymes on plasma bradykininogen (19).

The major difficulty in approaching the study of the structure of physalaemin was the scarcity of material, since about 15-18 mg of pure peptide were available at the end of the purification procedures. The high-voltage electrophoresis technique was of valuable help in overcoming the difficulties, as it permitted a good separation of the degradation products of very small samples of active peptide.

As already stated, the correctness of the proposed structure of physalaemin has been recently checked by comparing the biological activity, the physicochemical properties and the behavior toward chemical and biochemical degradative agents of the natural product with the properties of a synthetic preparation of the endecapeptide (20).

It has been pointed out that the behavior of physalaemin toward trypsin was in part quite unexpected. In an attempt to interpret the observed divergencies from the generally accepted schemes, we are now

carrying out experiments on trypsin fission of synthetic analogues of physalaemin. The anomalous cleavage by trypsin of two bonds, instead of one, in the physalaemin molecule explains the complete loss of activity of this polypeptide produced by trypsin. On the other hand, the normal cleavage of only one bond, between lysine and aspartic acid, in the eledoisin molecule, with consequent release of a fairly large heptapeptide fragment, explains why eledoisin retains 10–15% of its biological activity after trypsin digestion.

Some features of chymotryptic hydrolysis are also noteworthy. When the complete sequence of physalaemin was known, it became evident that chymotrypsin caused the fairly unusual quantitative splitting at the carboxyl bond of asparagine and a partial splitting of adjacent aromatic residues. Other workers have already observed similar behavior (21–24). It may be suggested that the slow cleavage of the phenylalanyl bond in physalaemin is due to inhibition by the adjacent free carboxyl produced by the faster rupture of the tyrosine bond.

Although eledoisin and physalaemin possess the same C-terminal tripeptide sequence, methioninamide is released rapidly and quantitatively from eledoisin, while it is very slowly and only in trace amounts liberated from physalaemin. This is probably due to the fact that, whereas the tetrapeptide, -Ile-Gly-Leu-Met-NH₂, which is first released from eledoisin, is susceptible to further easy hydrolysis by chymotrypsin, the tripeptide -Gly-Leu-Met-NH₂ produced by the major attack of the enzyme on the physalaemin molecule does not seem to constitute a good substrate for further hydrolysis. Experiments of digestion with chymotrypsin of the synthetic C-terminal tripeptide have confirmed this interpretation.

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