PROTEOLYTIC ACTIVITY OF PSEUDOTRYPSIN

V. KEIL-DLOUHÁ, N. ZYLBER, J.-M. IMHOFF, N.-T. TONG and B. KEIL
Institut de Chimie des Substances Naturelles, CNRS, 91-Gif-sur-Yvette, France

Received 7 July 1971

1. Introduction

Bovine α-trypsin differs from the single-chain β-trypsin by an interchain split between Lys_{131} and Ser_{132} [1]. Further degradation of α-trypsin leads to pseudotrypsin (ψ-trypsin) in which an additional bond is opened, between Lys_{176} and Asp_{177} [2]. This structural change occurs at the direct vicinity of Asp_{177} which represents the specificity site of trypsin for the binding of positively charged substrates.

Pseudotrypsin differs markedly from the α- and β-forms in its enzymatic properties. Its active center is only partially reactive: it reacts slowly in a stoichiometric ratio with DFP but resists to the substitution with TLCK. With NPGB, specific active site titrant, there is only a slow liberation of nitrophenol instead of an instantaneous "burst". Pseudotrypsin is devoid of any measurable amidase activity. It has only a poor affinity for positively charged ligands benzoyl arginine ethylester and benzamidine. On the other hand its affinity for acetyl tyrosine ethyl ester remains essentially unaltered [2].

The lack of amidase activity seems to indicate that in pseudotrypsin the proteolytic activity will also be absent. We wish to report on an observed proteolytic action and specificity of pseudotrypsin and on its ability to bind basic pancreatic trypsin inhibitor (BPTI).

Abbreviations:
DFP : diisopropyl fluorophosphate
TLCK: tosyl lysyl chloromethyl ketone
NPGB: *p*-nitrophenyl-*p'*-guanidinobenzoate
BPTI : basic pancreatic trypsin inhibitor.

2. Methods

The preparation of α-trypsin is described in a parallel study [3]. ψ-trypsin was prepared according to Smith and Shaw [2]. Autodigestion of α-trypsin was carried out for 7 hr under an atmosphere of nitrogen. The titration of the active center using NPGB [4] gave for α-trypsin a value of 90%. At the end of the autodigestion, this value was brought down to 46.9%. After two subsequent treatments with TLCK the mixture was submitted to chromatography on SE-Sephadex.

For determination of the N-terminal amino acids in ψ-trypsin dinitrofluorbenzene was used in combination with thin layer chromatography.

Crystalline glucagon (lot G 4250) was purchased from Sigma. Heptapeptide Gly–Phe–Phe–Tyr–Thr–Pro–Lys was the same sample as in the previous study [3]. BPTI was purified chromatographically [5].

Esterase activity assays were carried out in the same manner as described previously [6] with substrate concentration 0.1 M using a Metrohm Herisau type titrator.

Conditions of digestion of peptidic substrates and the methods for fractionations of the fragments by chromatography on Dowex 50 × 2, described in the parallel work [3] were followed without substantial change.

Gel filtration of the mixture of ψ-trypsin and BPTI was evaluated colorimetrically using BAPNA as assay substrate after precipitation of aliquots of the eluate with 5% trichloroacetic acid [7].

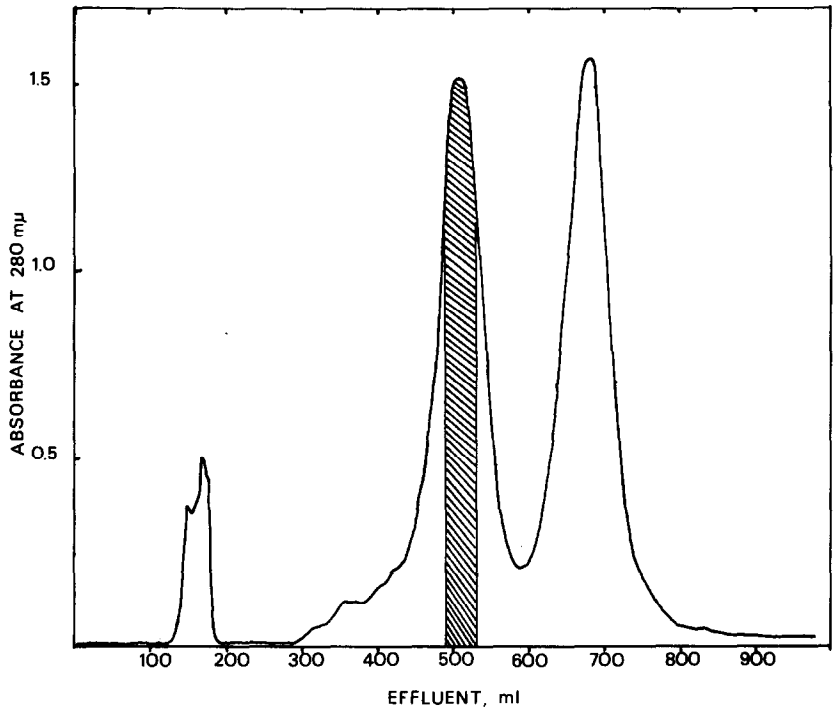

Fig. 1. Separation by ascending chromatography of ψ-trypsin and TLCK-α-trypsin on an SE-Sephadex C-50 column (1.5 × 150 cm) at 4° in 0.10 M, pH 7.1 tris-HCl buffer made 0.02 M in $CaCl_2$. The eluate was collected in 5 ml fractions at hour intervals, the central part of the ψ-trypsin peak was pooled.

3. Results

3.1. *Isolation and characterisation of ψ-trypsin*

Chromatography of the autolyzed α-trypsin gave the same pattern as that described by Smith and Shaw [2] (fig. 1). The peak, corresponding to ψ-trypsin, was well separated from the peak of TLCK-α-trypsin. In addition to isoleucine and serine which have been found as N-terminal groups in the parent α-trypsin [3], the presence of a third end group, aspartic acid, was detected in ψ-trypsin. During the reaction of ψ-trypsin with NPGB, a very slow evolution of nitrophenol was observed, instead of a burst characteristic for α-trypsin (fig. 3).

3.2. *Incubation of glucagon with ψ-trypsin and TLCK-α-trypsin and analysis of the resulting products*

Glucagon was incubated with ψ-trypsin or TLCK-α-trypsin under conditions described under fig. 2. At the end of the incubation with ψ-trypsin, all the suspension of glucagon disappeared, the small precipitate formed on subsequent acidification was removed by centrifugation. The chromatography of soluble products on Dowex 50 × 2 column gave seven peptide fractions (fig. 2).

Peptides were analysed directly after evaporation of the corresponding fraction or they were rechromatographed by paper chromatography before analysis. Recoveries of all isolated peptides are compared with the results of previous studies [3, 8, 9] in table 1.

Glucagon incubated under similar conditions with TLCK-α-trypsin remained insoluble. No detectable amount of peptidic material was observed after chromatography of the supernatant.

3.3. *Assay of chymotryptic-like activity with Gly–Phe–Phe–Tyr–Pro–Lys as substrate*

Incubation of the heptapeptide was carried out at pH 9.0 and 37° in parallel experiments with ψ-trypsin and with twice crystallized trypsin Worthington TLR

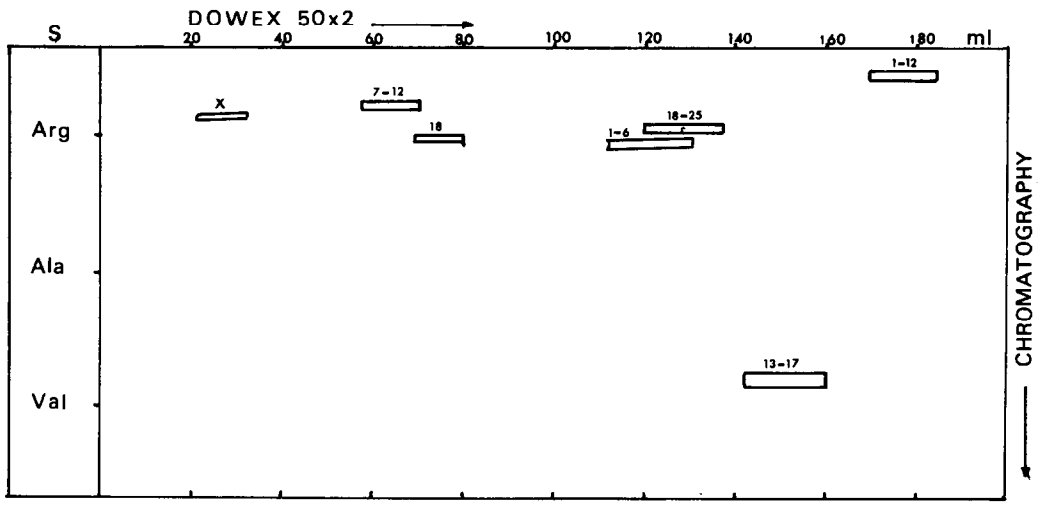

Fig. 2. Elution pattern of a ψ-trypsin hydrolysate of glucagon. The eluate from a Dowex 50 × 2 column (0.4 × 65 cm, 200–400 mesh) was collected in 1 ml fractions at 20 min intervals. Two subsequent gradients of pyridine acetate buffers (35 ml 0.15 M pH 3.0 – 35 ml 0.40 M pH 5.5; 50 ml 0.40 M pH 5.5 – 50 ml 0.80 M pH 7.0) were followed by 50 ml of 2 M pyridine pH 8.5. 0.4 ml of each fraction was evaporated for chromatography in butanol–pyridine–acetic acid–water. Ordinate – no. of fractions; abscissa (S) – standard amino acid mixture.

Table 1

Yields of peptides resulting from the digestion of glucagon by commercial trypsin and pure α-, β- and ψ-trypsin.

Trypsin	Incubation		Yield in %						Ref.
	hr	°	$His_1–Phe_6$	$Thr_7–Lys_{12}$	$Tyr_{13}–Arg_{17}$	Arg_{18}	$Ala_{19}–Trp_{25}$	$Leu_{26}–Thr_{29}$	
Worthington	50	25	30	55	95	80	30	35	[8]
							30		
Worthington	15	30	31	32	38	31	43	60	[9]
Worthington	15	30	12	16	50	50	44	42	[9]
			18						
α	50	25	61		47	83[b]	61		[3]
							10		
β	50	25	47		51	81[b]	68		[3]
							12		
ψ	50	25	16	6	30	23[b]	16	a	this paper
			12				8		

[a] Not determined quantitatively.
[b] Direct assay of the digestion mixture on the short column of the amino acid analyser.

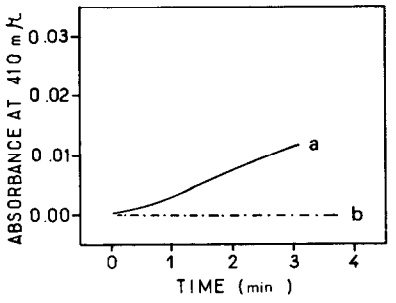

Fig. 3. Inhibition of the reaction between NPGB and ψ-trypsin by BPTI. (a) NPGB (1×10^{-4} M) and ψ-trypsin (1.5×10^{-6} M), time dependence of *p*-nitrophenol liberation, (b) the same, BPTI (6×10^{-6} M) added to ψ-trypsin 30 min before the assay.

to which 1% of α-chymotrypsin was added. Reaction mixtures were analysed by paper chromatography. No splitting of the heptapeptidic substrate by ψ-trypsin could be detected. In a parallel experiment, a contamination of α-chymotrypsin in commercial trypsin caused a total disappearance of the substrate and a formation of six peptides corresponding to the fragmentation at the sites of aromatic residues.

3.4. *Inhibition of catalytic action of ψ-trypsin by BPTI*

During the reaction of ψ-trypsin with the active site titrant NPGB a slow liberation of *p*-nitrophenol occurs. In the presence of a 4-fold molar excess of BPTI the reaction between ψ-trypsin and the active site titrant is inhibited (fig. 3).

Similarly the original esterolytic activities of ψ-trypsin towards benzoyl arginine ethyl ester (2.25 μeq/min/mg) and acetyl tyrosine ethyl ester (0.64 μeq/min/mg) were completely inhibited in the presence of BPTI.

3.5. *Direct interaction between ψ-trypsin and BPTI*

Gel filtration of a mixture of BPTI with ψ-trypsin in a molar ratio 2:1 shows two peaks of inhibitory activity, when aliquots of the eluate have been treated with trichloroacetic acid prior to activity assay (fig. 4). The first one corresponds to BPTI bound to ψ-trypsin, while the second represents the excess of BPTI.

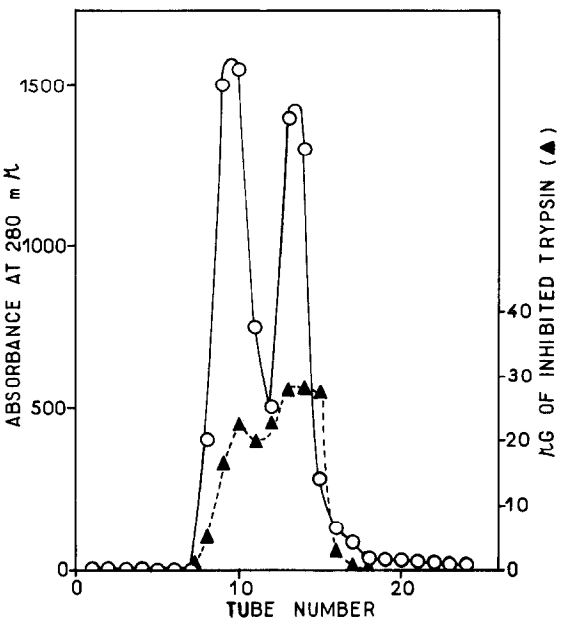

Fig. 4. Gel filtration of BPTI and ψ-trypsin complex on a Sephadex G-75 column (1 × 45 cm) in 0.05 M tris-HCl, 0.02 M $CaCl_2$ buffer pH 8.3 at 25°. 1.5 ml fractions were collected in hour intervals. △ - - - △, inhibition of β-trypsin by the samples after their treatment with 5% trichloroacetic acid.

4. Discussion

When discussing the observed degradation of glucagon by ψ-trypsin (fig. 2 and table 1) we have to take in careful consideration the possible objection, that our preparation of ψ-trypsin has been contaminated by α-trypsin from which it originates.

In a parallel study [3] we have shown, that pure α-trypsin cleaves in glucagon exclusively the bonds adjacent to three basic amino acid residues. The presence of α-trypsin, still surviving the treatment of the autolysate with TLCK, could therefore explain the cleavage at these sites, but not the degradation at the aromatic amino acid residues.

If active α-trypsin would be present in the TLCK-treated autolysate prior to the chromatography shown in fig. 1, it would contaminate according to its elution volume rather the peak of TLCK-α-trypsin than that of ψ-trypsin. The incubation experiment which we have performed with glucagon and

TLCK-α-trypsin resulting from the same column separation as ψ-trypsin, has shown that it was entirely inactive. This seems to eliminate the possible source of erroneous interpretation.

The additional cleavage of bonds adjacent to two aromatic amino acid residues is similar to that observed by other investigators [8, 9] during the digestion of glucagon by commercial samples of crystalline non-fractionated trypsin (table 1). To compare this additional activity of ψ-trypsin to that of α-chymotrypsin, we have submitted to their action a heptapeptide, which is highly sensitive to the action of chymotrypsin. No traces of chymotryptic activity could be detected in ψ-trypsin.

We therefore conclude, that both types of cleavages observed in glucagon, at the sites of basic and of certain aromatic residues, resulted from the activity intrinsic to ψ-trypsin.

The yield of fragments is lower than that produced by α- and β-trypsin under similar conditions [3]. Remarkably lower is the liberation of free Arg_{18}.

All this witnesses a relatively low affinity of ψ-trypsin for polypeptidic substrates. As regards its affinity for a polypeptidic inhibitor, BPTI, the specific interaction was proved in two independent ways: BPTI inhibits the hydrolytic action of ψ-trypsin on an active site titrant and two ester substrates, on the other hand it associates with ψ-trypsin as showed by the gel filtration experiment.

It can be concluded that whereas the cleavage of the first bond Lys_{130}–Ser_{131} in β-trypsin leading to α-trypsin did change neither the specificity nor other properties of trypsin [1, 3], the cleavage of the bond Lys_{176}–Asp_{177} in α-trypsin has a profound influence on the catalytic properties of the resulting ψ-trypsin. In addition to the changes described earlier in the original work on this enzyme [2], ψ-trypsin displays in contrast to the α- and β-form an ability to split a very limited number of bonds adjacent to the aromatic amino acid residues. Although the opening of the bond Lys_{176}–Asp_{177} in the trypsin molecule has lowered the affinity for polypeptidic substrate and broadened the specificity, it did not destroy the binding site for basic pancreatic trypsin inhibitor.

Acknowledgement

The authors thank Professor Edgard Lederer for his interest and support in the work.

References

[1] D.D. Schroeder and E. Shaw, J. Biol. Chem. 243 (1968) 2943.
[2] R.L. Smith and E. Shaw, J. Biol. Chem. 244 (1969) 4704.
[3] V. Keil-Dlouhá, N. Zylber, N.-T. Tong and B. Keil, FEBS Letters 16 (1971) 287.
[4] T. Chase, Jr. and E. Shaw, Biochem. Biophys. Res. Commun. 29 (1967) 508.
[5] V. Dlouhá, N. Neuwirthová, B. Meloun and F. Šorm, Collection Czech. Chem. Commun. 30 (1965) 1705.
[6] J.-M. Imhoff and V. Keil-Dlouhá, FEBS Letters 12 (1971) 345.
[7] V. Dlouhá, B. Keil and F. Šorm, Biochem. Biophys. Res. Commun. 31 (1968) 66.
[8] W.W. Bromer, A. Staub, L.G. Sinn and O.K. Behrens, J. Am. Chem. Soc. 79 (1957) 2801.
[9] S. Maroux, M. Rovery and P. Desnuelle, Biochim. Biophys. Acta 122 (1966) 147.