CLEAVAGE OF GLUCAGON BY α- AND β-TRYPSIN

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1. Introduction

Trypsin is one of the most frequently used proteolytic enzymes in the protein sequence studies. Although it hydrolyzes preferentially the peptide bonds involving the carboxyl groups of arginine and lysine residues in polypeptidic substrates, additional cleavage at the carboxyl groups of certain other amino acid residues has been observed in several cases [1–13]. Many procedures have been described to eliminate chymotrypsin present as a contaminant; rechromatography [4, 14, 15], electrophoresis [16], incubation or chromatography in 8 M urea [17, 18], incubation in dilute acid [19, see also 4, 20–23], partial desactivation by diisopropyl fluorophosphate [24, see also 25], specific desactivation of chymotrypsin by diphenyl carbamyl chloride [1], β-phenyl propionate [2] or by L-(1-tosylamido-2-phenyl) ethylchloromethyl ketone (TPCK) [26, see also 27].

Nevertheless, after total elimination of chymotrypsin there remained a chymotryptic-like activity. Previously reported evidence [28, 29] that this activity is intrinsic to the trypsin molecule, was later supported experimentally by many authors [2, 4, 6, 14, 18]. Trypsin was found to hydrolyze not only typical synthetic substrates of chymotrypsin, like acetyl tyrosine ethyl ester, but it catalyzed cleavages of certain polypeptides containing aromatic amino acid residues with greater selectivity than chymotrypsin itself. Thus only one out of six bonds adjacent to aromatic residues were split in chain C of α-chymotrypsin [4], two out of five in glucagon [2, 4] in addition to the usual tryptic cleavage.

The improvement in fractionation techniques has made it possible to show that all preparations of crystalline trypsin which have been used for all the abovementioned studies represented mixtures. Pure single-chain β-trypsin and the double-chain α-trypsin can be obtained now by chromatography of crystalline trypsin on a SE-Sephadex column [30]. This prompted us to study the specificity of the two individual enzyme forms, α- and β-trypsin on polypeptidic substrates. Glucagon was used as substrate because in this case, according to two previous independent studies [2, 4], crystalline trypsin shows pronounced additional chymotryptic-like specificity. Insulin B-chain fragment Gly_{23}–Lys_{29} was used because of its high content in aromatic amino acid residues.

2. Methods

α- and β-trypsin were isolated from commercial trypsin Worthington (twice crystallized, dialyzed salt-free, lyophilized, 196 U/mg, TRL) according to the procedure of Schroeder and Shaw [30]. The identity of the products obtained with those described [30] was proved by the position of peaks on the elution diagram and by end group analyses. One N-terminal isoleucine was obtained per mole of β-trypsin, one serine was found in addition to isoleucine in α-trypsin. The molarity of the active enzymes was determined in the lyophilized preparations by titration of the active site. It gave a value of 90% for α-trypsin and 82% for β-trypsin. We have revised the values of maximal rates of esterolytic activity, which we had observed during the
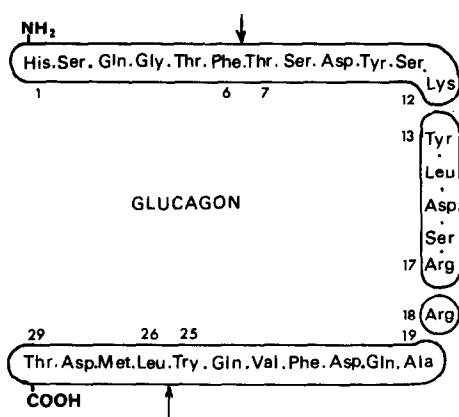
Fig. 1. Fragmentation of glucagon by trypsin. The peptides 1–12, 13–17, 18 and 19–29 result from specific trypsin digestion. Arrows indicate additional cleavages observed when crystalline trypsin has been used [2, 4].
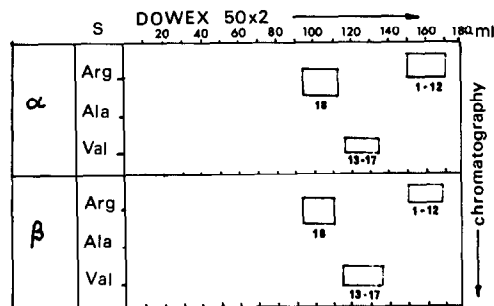

Fig. 2. Elution pattern of α- and β-tryptic hydrolysates of glucagon. The eluate from a Dowex 50 × 2 column (0.4 × 65 cm, 200 – 400 mesh) was collected in 1 ml fractions at 20 min intervals. Two subsequent gradients of pyridine acetate buffers (50 ml 0.15 M pH 3.0 –50 ml 0.4 M pH 5.5; 35 ml 0.40 M pH 5.5–35 ml 0.80 M pH 7.0) were followed by 50 ml of 2% ammonia. 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 α- and β-trypsin (50 hr at 25°).

Peptide	α	β
$His_1 - Lys_{12}$	61	47
$Tyr_{13} - Arg_{17}$	47	51
Arg_{18}	83	81
$Arg_{18} - Thr_{29}$	10	12
$Ala_{19} - Thr_{29}$	61	68

previous study [31]. α- and β-trypsin were found equally active towards benzoyl arginine ethyl ester (spec. activity 72 and 75 μeq/min/mg, respectively). Both forms of trypsin were found active towards acetyl tyrosine ethyl ester, although the β-derivative possessed slightly higher activity (42 μeq/min/mg) than the α-form (29 μeq/min/mg).

Crystalline glucagon was a product of Sigma (lot G 4250).

Heptapeptide Gly–Phe–Phe–Tyr–Thr–Pro–Lys was isolated from an α-tryptic hydrolysate of crystalline insulin chromatographically [23].

2.1. Substrate digestion

Glucagon (1.29 μM) was incubated with pure α- or β-trypsin (0.013 μM) in 5 ml 0.1 M ammonium

carbonate made 0.02 M in calcium chloride, at pH 8.0 and 25° for 50 hr. The digestion mixture was then acidified by addition of acetic acid to pH 3.0 and lyophilized.

The heptapeptidic substrate was digested with pure α- and β-trypsin, with twice crystallized trypsin Worthington TRL and with the same enzyme to which 1% crystalline chymotrypsin had been added. The digestion was carried out in the same buffer solution at pH 9.0 and 37° for 16 hr at an enzyme-substrate molar ratio of 1:80. The digest was acidified with HCl, taken to dryness and chromatographed in solvent system n-butanol–pyridine–acetic acid–water (15:10:3:12).

2.2. Fractionation of glucagon hydrolysate

The lyophilized digest was suspended in 0.1 M pyridine-formate buffer pH 2.85. The insoluble part was centrifuged off and washed twice with the same buffer. The precipitate was dissolved in dilute ammonium hydroxide and aliquots were withdrawn for total hydrolysis and quantitative amino acid analysis.

The combined supernatants from the centrifugation step were chromatographed on a Dowex 50 × 2 column equilibrated with 0.1 M pyridine formate buffer at pH 3.0 . The peptides were eluted

with pyridine acetate buffers of increasing gradient of molarity and pH. The eluate was analyzed by paper chromatography of aliquots of each fraction in the system n-butanol—pyridine—acetic acid—water (15:10:3:12). Quantitative analyses of the peptides from the pooled fractions were carried out by the method of Spackmann et al. [32].

3. Results and discussion

Fig. 1 shows the four fragments which should result from a tryptic hydrolysis of glucagon. With an unfractionated trypsin this pattern of cleavage has been observed only when short hydrolysis times have been used [2]. With increasing time additional desintegration has been observed at two sites next to aromatic residues [2, 4]. The treatment of crystalline trypsin with TPCK has partially removed this chymotrypsin-like cleavage only at one of the two sites [4].

The patterns of fragments resulting from the digestion of glucagon with pure α- and β-trypsin were identical. Preliminary analyses using the fingerprint technique and a detailed fractionation by column chromatography (fig. 2) gave the same pattern. The acid-soluble fraction of the digest was composed only of the N-terminal fragment His_1-Lys_{12}, the pentapeptide $Tyr_{13}-Arg_{17}$ and free arginine. The acid-insoluble fraction contained predominantly the C-terminal fragments $Ala_{19}-Thr_{29}$ and $Arg_{18}-Thr_{29}$. Recoveries of all peptides isolated are compared in table 1. This comparison indicates, that pure α- and β-trypsin are devoid of additional chymotrypsin-like activity even with long hydrolysis time. Further experience must show, if this sharp specificity is maintained in the case of other polypeptidic substrates.

It has been demonstrated on both B-chain of insulin [4, 26] and on the heptapeptide Gly—Phe—–Phe—Tyr—Thr—Pro—Lys [26], that they are cleaved at the aromatic residues only when trypsin is "contaminated with traces of an enzyme possessing the same specificity as chymotrypsin" [26]. Our results confirm these observations. α- and β-trypsin are without effect on the heptapeptide; addition of α-chymotrypsin causes its prompt hydrolysis at the sites of aromatic residues.

Although α- and β-trypsin retain the ability of crys-

talline trypsin to hydrolyse acetyl tyrosine ethyl ester, they no more show the tendency to split glucagon at the carboxyl of aromatic amino acid residues; this type of cleavage may therefore be due to some other active component of commercial crystalline trypsin. One of the possible explanations is discussed in a parallel study [33].

Acknowledgement

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