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New low-molecular inhibitors of pancreatic elastase with possible in vivo application: Alkylamides of N-acylated tripeptides

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Summary. Out of a series of alkylamides of N-acylated tripeptides, Glt-$(Ala)_2$-Pro-NH-Et and Glt-$(Ala)_3$-NH-Pr were found to be potent inhibitors of porcine and human pancreatic elastase, and because they are free of toxic groups they might be considered for in vivo application.

Hassall et al.[1] introduced alkylamides of N-alkyl- and N-cycloalkanoyl-dipeptides as novel pancreatic elastase inhibitors with potential for action in vivo. Their design does not, however, take into account some new data on elastase-substrate interaction. In previous reports[2-4] we established the prerequisites for a synthetic substrate for pancreatic elastase. This knowledge was used in the synthesis of alkylamides of N-acylated tripeptides. Their inhibitory effect on pancreatic elastase of various origins is reported.

Materials and methods. Inhibitors. The alkylamide residue at the P_1-position (nomenclature of Schechter and Berger[5]) was -ethylamide (NH-Et) or -propylamide (NH-Pr). The acyl residues at the P_5-position included acetyl- (Ac), maleyl- (Mal), succinyl- (Suc) and glutaryl- (Glt). The amino acid sequence at the P_4-P_3-P_2 position was alanine-alanine-alanine (Ala_3) or alanine-alanine-proline (Ala_2-Pro). The synthesis has been described elsewhere[6]. The series of inhibitors is surveyed in the table.

Substrates. Succinyl-tetraalanine-4-nitroanilide (Suc-Ala_4-NAn) and glutaryl-tetraalanine-4-nitroanilide (Glt-Ala_4-NAn), synthesized according to our earlier method[3] were used.

Enzyme. 1. Porcine pancreatic elastase (E 1) Serva (cat. No.20929) was dissolved in 1 mM AcOH. The enzyme concentration was 15.5 nM. 2. Human duodenal contents (pH 7.2-7.5) were aspirated separately from gastric juice after cholecystokinin stimulation. Elastase (E 2) was isolated by chromatography on a DEAE-Sephadex A-50 column[7]. 3. Human pancreatic juice after secretin-cholecystokinin stimulation was obtained by cannulation of the main pancreatic duct (E 3). The lyophilized sample was dissolved in 0.1 M Tris buffer pH 8.2 with Ca^{2+}-ions (5 mM). Zymogens were activated with trypsin (0.3 mM).

Assay of elastase inhibition. The incubation medium consisted of 0.1 ml substrate solution (in dimethylsulphoxide), 1.3 ml 0.1 M Tris buffer pH 8.0, 0.05 ml inhibitor in Tris buffer, and 0.05 ml elastase solution. The enzymic activity was measured at 25 °C by monitoring continuously the split-off 4-nitroaniline (410 nm). K_m-values of Suc-$(Ala)_4$-Nan and Glt-$(Ala)_4$-NAn were estimated at 3 substrate concentrations (0.3125-0.625-1.25 mM) by a Lineweaver-Burk plot. Inhibition constants (K_i) were determined at 2 inhibitor concentrations using a Lineweaver-Burk or Dixon plot (for E 1) and at 1 inhibitor concentration using the intercept with 1/V which was determined by a Lineweaver-Burk plot (for E 2 and E 3).

Results and discussion. K_m-values of Suc-$(Ala)_4$-NAn and Glt-$(Ala)_4$-NAn amounted to 0.356 mM and 0.314 mM respectively. The inhibition constants (K_i) of ethyl- and propylamides of acylated trialanine- and dialanine-proline peptides are summarized in the table. These substances are distinctly more potent inhibitors than the alkylamides of N-alkyl or N-cycloalkanyol-dipeptides introduced by Hassall et al.[1]. In contrast to these authors, we found that the inhibitory effect is further increased, if ethylamide at P_1 is replaced by propylamide. This finding supports our earlier opinion[3], that a substrate with α-aminobutyric acid at the P_1-position may be very suitable for pancreatic elastase. Proline at the P_2-position is superior to alanine both in substrates[4,8] and inhibitors. From the various acyl residues at P_5-position, the presence of an anionic residue of dicarboxylic acid, particularly glutaryl-, appears to be most favorable. These findings hold true for porcine as well as

Inhibition constants (K_i) of ethyl- and propylamides of N-acylated trialanine and dialanine-proline-peptides

Inhibitor	K_i (μM) Suc-$(Ala)_4$-NAn			Glt-$(Ala)_4$-NAn		
	E1	E2	E3	E1	E2	E3
Ac-$(Ala)_3$-NH-Et	19	238	69	21	123	117
Mal-$(Ala)_3$-NH-Et	35	87	69	30	73	117
Suc-$(Ala)_3$-NH-Et	20	76	29	29	89	47
Glt-$(Ala)_3$-NH-Et	8.8	15	7.4	8.4	19	19
Suc-$(Ala)_2$-Pro-NH-Et	6.0	14	23	4.0	19	17
Glt-$(Ala)_2$-Pro-NH-Et	1.6	10	2.9	2.0	14	3.8
Suc-$(Ala)_3$-NH-Pr	6.8	12	13	4.5	21	14
Glt-$(Ala)_3$-NH-Pr	2.5	11	3.8	2.5	7.2	4.5

Source of enzyme: E1, porcine pancreatic elastase; E2, elastase of human duodenal contents after cholecystokinin stimulation; E3, elastase of human pancreatic juice cannulated via the main pancreatic duct (stimulation: secretin-cholecystokinin). Substrates: Suc-$(Ala)_4$-NAn and Glt-$(Ala)_4$-NAn.

human elastase. The lower effect of synthetic inhibitors on elastase of human duodenal and pancreatic juice is probably due to the presence of natural inhibitors. The K_i-values of Glt-$(Ala)_2$-Pro-NH-Et and Glt-$(Ala)_3$-NH-Pr appear to be particularly promising and they may be further improved by combining the preferences of proline at the P_2- and propylamide at the P_1-position. At the same time, these inhibitors are free of any toxic group and it is therefore possible that they might be used in vivo.

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A simple method for the purification of the carcinoembryonic antigen without the use of perchloric acid*

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Summary. A method for the purification of the carcinoembryonic antigen CEA without perchloric acid extraction is described. Addition of a synthetic polycarbonic acid precipitates proteins in serum or aqueous tumor extracts while CEA remains in the supernatant.

The perchloric acid extraction method according to Krupey, Gold and Freedman[2], or modifications of it[3], are mainly used for the isolation of the carcinoembryonic antigen (CEA), a glycoprotein with molecular weight of about 200,000 daltons. Perchloric acid extraction is also used to pretreat patient samples before performance of the CEA radioimmunoassay. CEA determinations have found applications in the diagnosis and management of patients with neoplastic disease.

During the perchloric acid extraction CEA is exposed to a low pH and to oxidative conditions, which can result in loss of activity[4]. Efforts have been made to replace this extraction by milder methods. Techniques using lithium diiodosalicylate[5], ethanol[6,7] and extraction with buffer solutions[8] have been described. Further purification is performed by applying chromatographic techniques to these extracts.

The perchloric acid extraction of patient samples before performance of the radioimmunoassay permits precipitation of interfering plasma proteins. It also gives shorter immunological incubation times and often a higher sensitivity of the assay, when compared to direct assays without pretreatment of the samples.

We have found that the perchloric acid extraction can be replaced by treating patient samples before performing the immunological assay with an expandable, insoluble polycarbonic acid. This polycarbonic acid can also be used as the first purification step for the isolation of CEA from aqueous tumor extracts. This insoluble polycarbonic acid is obtained by crosslinking maleic anhydride resins with ethylenediamine and by hydrolysis of the unreacted anhydride groups to the free carboxylic groups. If the polycarbonic acid is added to plasma, serum or a tumor extract, most of the proteins are precipitated while CEA remains in solution.

Materials and methods. Deproteinization of serum samples. The crosslinked polycarbonic acid was prepared by methods that will be published later[9]. The resins used as starting materials were obtained from Monsanto Company, St. Louis, USA (EMA resins) and from GAF Corporation, New York, USA (Gantrez AN resins). 5 g of the dry product were suspended in about 70 ml of distilled water and treated with a Potter homogenizer to obtain a very fine aqueous suspension, which was then adjusted to 100 ml. This suspension is stable at 2–8 °C for several weeks. The final 1% suspension used for the deproteinization experiments was obtained by diluting 5-fold the 5% suspension with an ammonium acetate buffer 0.01 M, pH 6.8.

A deproteinization curve with serum was established according to figure 1: Radiolabeled ^{125}I-CEA (5–10,000 cpm per 100 µl serum) was added to human serum. Aliquots of

Typical purification of CEA from liver metastasis

Purification step	Total proteins g	Protein %	Total CEA acitivity mg	CEA %	Specific activity mg CEA/mg protein
Liver metastasis	1160.0	–	–	–	–
H_2O extract	44.240	100	356	100	0.008
Supernatant after deproteinization	1.120	2.5	85.7	24	0.076
Sephadex G 200 gelfiltration:					
Pool 1 lyophilized	0.125	0.28	50	14	0.4
Pool 2 lyophilized	0.050	0.11	11	3	0.22