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

100 µl of this serum were then introduced into each of 10 reagent tubes and diluted with the ammonium acetate buffer 0.01 M, pH 6.8 in such a way that the final volume, after addition of increasing amounts of the polymer suspension in each tube, reached 1 ml. After dilution of the serum samples with the ammonium acetate buffer, the appropriate volumes of the suspension were added dropwise to each tube with vigorous mixing with a vibromixer (type Vortex). After 15 min, the tubes were centrifuged and the supernatant separated from the precipitate. The protein concentration in the supernatant was measured with an appropriate method[10] and the radioactivity was determined with a γ-counter. CEA activities were measured with the CEA-Roche RIA.

Purification of CEA from a tumor extract. 500 g tumor tissue (fresh liver metastasis of a primary colonic adenocarcinoma) was extracted with 1 l of distilled water by homogenizing the tissue in a Waring blender at 4 °C. The homogenate was then centrifuged, and the protein concentration determined in the supernatant. A 10% suspension of the crosslinked polycarbonic acid was added (corresponding to an equal dry weight of the polycarbonic acid and the protein content), stirred for 15 min and centrifuged. The clear supernatant was concentrated to 70–80 ml at about 40 °C under reduced pressure in a rotary evaporator. This solution was again centrifuged if a precipitate was formed after standing at 4 °C for about 20 h.

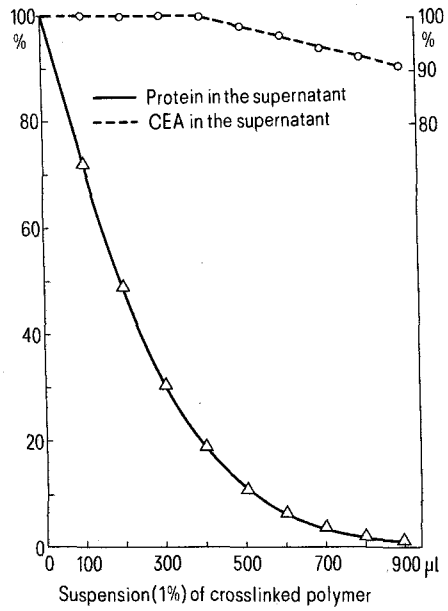

Figure 1. Deproteinization curve of human serum containing ^{125}I-CEA.

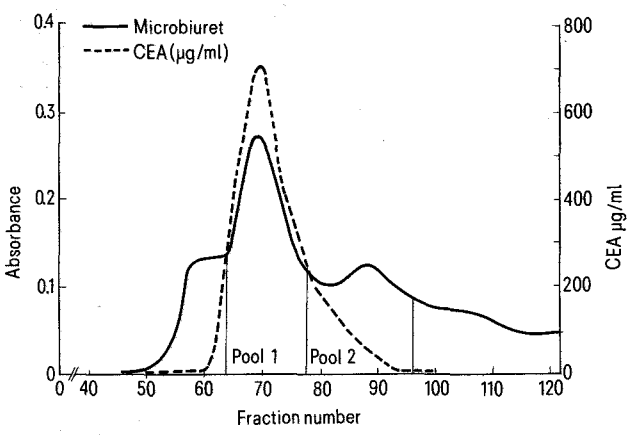

Figure 2. Tumor extract. Gel filtration on Sephadex G 200 of CEA after deproteinization with the crosslinked polycarbonic acid. Column (5 × 100 cm), sodium phosphate buffer 0.05 M, pH 5.0. Flow rate: 50 ml/h; fractions: 10 ml; temperature 4 °C.

Results and discussion. The treatment of biological fluids with a synthetic, crosslinked polycarbonic acid is a very efficient deproteinization method. It is useful as pretreatment of cancer patient samples before the performance of the CEA-test.

Figure 1 shows the effect of adding increasing amounts of the polymer to human serum containing ^{125}I-labeled CEA. When 900 µl of a 1% polymer suspension was added to a 100-µl serum sample, the soluble protein content was decreased by 97% or more, whereas the loss of CEA was smaller than 20%. In order to carry out deproteinization experiments it is important to use freshly labeled ^{125}I-CEA, since radioactive degradation products may falsify the results.

This procedure is a very convenient method for the purification of CEA from tumor extracts under physiological conditions. The protein content of aqueous tumor extracts can be drastically reduced and the overall operations for the isolation of CEA are much simpler and less time-consuming than with the perchloric acid extraction or other methods. Additional purification has been achieved by gel filtration on Sephadex G 200. Depending on the CEA content of the tumors, the quality of the CEA preparations after this step is sufficient for many applications. We have labeled such CEA preparations with ^{125}I, and used them as standards, which have given practically identical results when compared with reagents of the 'CEA-Roche Test'. Some of these CEA preparations served as antigens for the production of monoclonal antibodies.

The table summarizes results of a typical purification using this method. The combined procedures of pretreatment of tumor extracts with a polymer suspension followed by Sephadex G 200 gel filtration often yield CEA preparations with a specific activity of 0.4 mg CEA/mg protein or better. The elution diagram of a Sephadex G 200 gel filtration is presented in figure 2. The polyacrylamide gel electrophoresis and the ultracentrifuge pattern (not shown) of selected CEA fractions or pools after Sephadex G 200 gel filtration have often shown high homogeneity, depending on the quality of the tumor tissue used as starting material. In order to achieve a specific activity of 1, however, it is necessary to add another selective chromatography step.

The method described is very mild, rapid, easy to carry out, and gives an efficient purification of CEA from tumor extracts.

* Dedicated to Prof. Dr Walter Boguth on the occasion of his 65th birthday.

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Ascidian sperm chymotrypsin-like enzyme; participation in fertilization

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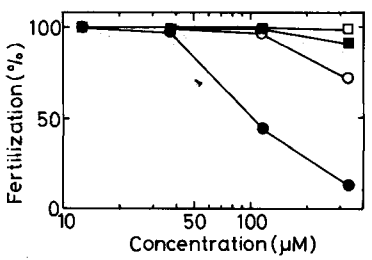
Summary. The action of a chymotrypsin-like enzyme from sperm extract from the ascidian *Halocynthia roretzi* was studied using several substrates. It was found that the most susceptible substrate had the most powerful inhibitory effect on fertilization in this animal. Among the substrates, the order of susceptibility coincided with the order of inhibitory ability, which indicates that the enzyme is involved in the fertilization process.

It has been proposed that sperm acrosin[EC 3.4.21.10], an acrosomal trypsin-like enzyme, plays a key role in the penetration of sperm through the zona pellucida of the ovum of mammals[3]. In marine invertebrates, however, little evidence has been reported[4–6] for the involvement of sperm proteases in fertilization. In a previous paper[7], we reported that chymotrypsin-like and trypsin-like enzymes may be indispensable for the sperm of the ascidian, *Halocynthia roretzi* to penetrate through the egg investment. Now we present further evidence for the involvement in fertilization of a chymotrypsin-like enzyme in sperm extract of the ascidian.

Materials and methods. Preparation of sperm and eggs from the ascidian, *H. roretzi*, type C[8], and the assay of fertilization in the presence or absence of various substrates were described previously[7,9]. Sperm suspension (1.6×10^{10} spermatozoa/ml) stored at −20 °C was thawed and homogenized with a Teflon homogenizer at 0 °C in a equal volume of 0.46 M NaCl-10 mM $CaCl_2$-50 ml $MgCl_2$-10 mM KCl buffered with 50 mM Tris-HCl (pH 8.0). After centrifugation (18,000 × g, 60 min), the resulting supernatant was employed as an enzyme preparation.

Results and discussion. Chymotrypsin-like activity in the enzyme preparation was examined with 4 peptidyl-4-methylcoumaryl-7-amide (MCA) substrates. As shown in the table, succinyl-Leu-Leu-Val-Tyr-MCA (Peptide Institute, Japan) was most susceptible to the enzyme, and acetyl-Ala-Ala-Tyr-MCA (Bachem, Switzerland), succinyl-Ala-Ala-Pro-Phe-MCA (Peptide Institute) and glutaryl-Gly-Gly-Phe-MCA (Bachem) followed in this order. The results suggest that the S_1 site[10] of the enzyme fits into the Tyr residue rather than the Phe residue of the substrate, and the S_2, S_3, or S_4 sites into the residue with a hydrophobic or branched side chain. Succinyl-Leu-Leu-Val-Tyr-MCA-hydrolyzing activity was found to be markedly inhibited with 0.1 mM chymostatin (34% inhibition), 1 mM phenylmethanesulfonyl fluoride (25%) and 1 mM diisopropylphosphorofluoridate (17%), when assayed after preincubation at 25 °C for 30 min, but not significantly with 1 mM EGTA, 0.1 mM leupeptin, 0.1 mM bestatin, 1 mM TPCK, 1 mM TLCK, and 0.01 mM proteinaceous protease inhibitors, such as soybean trypsin inhibitor (Sigma, USA), lima bean trypsin inhibitor (Sigma) and *Streptomyces* subtilisin inhibitor. The activity showed a pH optimum between 8.5 and 9.0. Thus, this enzyme is a chymotrypsin-like protease, different from the acrosin-like enzyme reported previously[9].

Inhibition of fertilization with the substrates used above was then examined (fig.). The results show that succinyl-Leu-Leu-Val-Tyr-MCA, the most susceptible substrate for



Inhibition of fertilization with various substrates. 50 μl of sperm suspension was added to sea water buffered with 50 mM Tris-HCl (pH 8.0) containing in a final volume of 1-ml about 100 eggs in the presence or absence of substrate. After standing for 30 min at about 13 °C, percentage of fertilization was estimated on the basis of chorion elevation. ●, Succinyl-Leu-Leu-Val-Tyr-MCA; ○, acetyl-Ala-Ala-Tyr-MCA; □, succinyl-Ala-Ala-Pro-Phe-MCA; ■, glutaryl-Gly-Gly-Phe-MCA. 7-Amino-4-methylcoumarin, one of the products of hydrolysis, showed no inhibitory activity toward fertilization at the concentration of 0.33 mM.

Substrate specificity of chymotrypsin-like enzyme in sperm extract

Substrate P_5 P_4 P_3 P_2 P_1 P_1'	Activity ($\times 10^{-11}$ mU/spermatozoon)	% Activity
Suc-Leu-Leu-Val-Tyr-MCA	6.3	100
Ac- Ala-Ala-Tyr-MCA	1.7	27
Suc-Ala- Ala-Pro-Phe-MCA	0.40	6
Glt-Gly-Gly-Phe-MCA	0.13	2

Enzymatic activity was measured by fluorophotometrical determination (excitation, 380 nm; emission, 460 nm) of the generation of 7-amino-4-methylcoumarin at 25 °C in the mixture of 0.1 mM substrate solution (0.5 ml) in 50 mM Tris-HCl (pH 8.0) containing 10 mM $CaCl_2$ and the enzyme solution (20 μl). One unit of activity is defined as the amount of enzyme that hydrolyzes 1 μmole of the substrate per min. The number of spermatozoa was counted under a microscope using a hemacytometer. Abbreviations used are: Suc, succinyl; Ac, acetyl; Glt, glutaryl; MCA, 4-methylcoumaryl-7-amide.