Proenzyme therapy of sarcoma S-180 and melanoma

B16-F10

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Summary

The aim of this study was to evaluate the effectiveness of individual (inactive) proenzymes and mixtures thereof in cancer treatment and to compare this treatment with more frequently used therapy based on active proteases. Experiments focused on explanation of possible mechanisms of proenzyme action against tumors are included.

Proenzyme therapy of sarcoma S-180 significantly reduced tumor growth and prolonged survival of mice. The effect of trypsinogen and chymotrypsinogen was synergistic.

Proenzyme therapy of melanoma B16-F10 bearing mice reduced both tumor growth and prevalence of metastases. Active enzyme based therapy of melanoma B16-F10 was less effective. Severe combined immunodeficiency (SCID) mice bearing sarcoma S-180 did not respond to the proenzyme therapy, indicating that the effect of this therapy is dependent on fully developed acquired immunity. Measured decreased levels of TGF-β and increased amount of alpha-2 macroglobulin in serum contributed to the elucidation of cancer treatment mechanism.

Proenzyme therapy based on administration of a mixture of trypsinogen and chymotrypsinogen is effective in cancer treatment.

Keywords: proenzyme therapy; proteases; sarcoma; melanoma; cancer therapy
INTRODUCTION

The beginnings of systemic pancreatic enzyme therapy of cancer date back to the early twentieth century. John Beard (1857-1924) noted a resemblance between embryonic/fetal trophoblastic cells and cancer cells and hypothesized that the growth of the former was eventually blocked by products of the fetal pancreas which becomes functional at about 56 days gestation. He further suggested that pancreatic enzymes were responsible for this ‘anti-trophoblast’ effect. Therefore, he proposed a new cancer therapy based on the administration of fresh pancreatic extracts. He called this treatment “trypsin therapy”, assuming that the pancreatic protease trypsin was the major active ingredient when combined with pancreatic amylase as a supplemental component (Beard 1911). This therapy had a wide range of successful followers (Cleaves 1906; Golley 1906; Cambell 1907; Golley 1909) but some scientists were not able to repeat the curative effect (Hald 1907; Bainbridge 1909) and trypsin therapy then fell out of use.

We assume that Beard’s early results could not be repeated by later investigators due to Beard’s incorrect assumption that trypsin is the active ingredient. While Beard pointed out the need to use fresh pancreatic extracts (containing also mainly trypsinogen, chymotrypsinogen and amylase), the investigators using trypsin in isolation would not observe any effect. In later work, Wald and collaborators achieved significant reduction in metastases using active proteases in mice intracutaneously inoculated with Lewis lung carcinoma (Wald et al. 1998) and B16 melanoma (Wald et al. 2001). In both cases, the enzyme mixture (trypsin, chymotrypsin and papain) was administered per rectum twice a day but the bioavailability of these proteases might be questioned.

The use of proenzymes in cancer therapy was first described by Trnka et al. (1999) and Novak and Trnka (2005). In vitro experiments showed inhibitory effects on the migration
and motility of cancer cells. *In vivo* experiments using mixtures of trypsinogen and amylase administered subcutaneously proved effective against methylcholanthrene-induced tumors and significantly reduced B16 melanoma metastases. The effects were achieved using trypsinogen concentrations ten or more times lower than that of the active enzymes used by Wald et al. (1998; 2001). This may reflect the selective conversion of proenzymes to the active form near the surface of tumor cells (Novak and Trnka 2005).

In the present investigations, we chose a relatively slow growing transplantable sarcoma primarily because we intended to study long term treatment of transplanted tumors. Sarcoma S-180, with low metastatic potential (Jones et al. 1939; Deodhar 1971) is a good model for this purpose. As the second model we used fast growing melanoma B16-F10. This model allowed us to evaluate the occurrence of metastases. We studied both the effect of proenzymes and that of a mixture of active proteases.

**MATERIALS AND METHODS**

*Enzymes.* Bovine trypsinogen, bovine alpha-chymotrypsinogen A, bovine trypsin, bovine alpha-chymotrypsin, and alpha-amylase from Bacillus sp. were obtained from Sigma.

*Mice.* SPF BALB/c and C57BL/6 mice were obtained from Charles River Laboratories (Sulzfeld, Germany). BALB/c and C57BL/6 mice were housed in plastic cages with wood-chip bedding situated in a specific-pathogen free room with a constant temperature of 22 °C and a relative humidity of 65%. SCID mice (C.B17/1cr-scid) of the BALB/c background were originally obtained from Charles River Laboratories (Sulzfeld, Germany) and were housed in
plastic cages with sterilized wood-chip bedding situated in flexible film isolators (BEM
Znojmo, Czech Republic) with high-efficiency particulate air filters. All mice were 18-20g.
Pellet diet and water were sterilized. All mice were housed in a 12/12-hour photoperiod
environment with free access to food and water.

Cells. Murine cell lines sarcoma S-180 and melanoma B16-F10 (both donated by Prof.
Rihova, Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague) were
used. The cells were cultivated in the same conditions - RPMI 1640 with 20 mM HEPES and
L-glutamine (Sigma) supplemented with 10% foetal calf serum (FCS) ≤ 10 EU/ml endotoxin
(Sigma), and Antibiotic/Antimycotic Solution (PAA). The cells were maintained at 37° C in
humidified air with 5% carbon dioxide.

Tumor transplantation. 4 x 10^5 S-180 or B16-F10 cells in 0.1 ml RPMI without serum per
mouse were inoculated subcutaneously (s.c.) in a shaved area on the right flank. We used
BALB/c and SCID mice for transplantation of S-180 and C57BL/6 for B16-F10 cells.

Treatment. Therapeutic preparations (proenzymes and enzymes dissolved in saline), sterilized
by filtration and stored frozen were injected daily i.m. in the semitendinosus muscle of the left
hind leg (0.1 ml per mouse). Doses correspond to original treatment (Beard 1911) reevaluated

Evaluation of treatment. Tumors were measured twice a week using calliper. Volume was
calculated according to the method of Inaba et al. (1986) using formula V = \pi/6 AB^2 (A
denotes the largest dimension of tumor mass and B stands for the smallest dimension).
Photographic documentation of tumor development and of side effects was also performed.

All dead mice were dissected.

*Lung metastases.* In melanoma B16-F10 experiments, all lungs were examined with the aid of a dissecting microscope. The presence of metastases (black points) was evaluated.

*Histology.* Tumors, liver, kidney, spleen and lungs were fixed by 4% neutral solution of formaldehyde. Paraffin blocks were prepared. Sections were stained by hematoxylin/eosin.

**Determination of total TGF-β in serum.** We used ELISA READY-SET-GO! Human/Mouse TFG-beta 1 kit (eBIOSCIENCE).

**Determination of trypsin activity.** Method developed by Erlanger et al. (1961) was used. This method uses N-alpha-benzoyl-DL-Arg-p-nitroanilid (BAPNA, Sigma) as a substrate.

*Proenzyme therapy of sarcoma S-180 bearing mice.* 40 female BALB/c mice were injected with sarcoma S-180 cells. Mice were randomly divided into 4 groups.

Group TG+CHG+A (9 mice): 7500 BAEE units of trypsinogen (after activation) + 31 BTEE units of alpha-chymotrypsinogen A(after activation) + 820 maltose units of alpha-amylase per ml in saline.

Group TG+A (9 mice): 15000 BAEE units of trypsinogen (after activation) + 820 maltose units of alpha-amylase per ml in saline.

Group CHG+A (10 mice): 62 BTEE units of alpha-chymotrypsinogen A(after activation) + 820 maltose units of alpha-amylase per ml in saline.

Group Control (10 mice): saline, control group.
The 2 remaining mice died during or shortly after tumor injection and were not considered in the analysis.

All solutions (0.1 ml per mouse) were injected daily from the fourth day after tumor cell transplantation. Mice were observed daily for 100 days. Tumor volumes were measured twice a week.

Proenzyme and enzyme therapy of melanoma B16-F10 bearing mice. 55 female C57BL/6 mice were injected with melanoma B16-F10 cells. All mice were monitored to document tumor onset. On day eleven after transplantation the mice were randomly divided into 4 groups. The therapy was started on the same day. Mice received daily doses of 0.1 ml of a solution composed as follows:

Group P “proenzyme therapy” (14 mice): 7500 BAEE units of trypsinogen (after activation) + 31 BTEE units of alpha-chymotrypsinogen A(after activation) + 820 maltose units of alpha-amylase per ml in saline.

Group E=P “enzyme therapy” (14 mice): 7500 BAEE units of trypsin + 31 BTEE units of alpha-chymotrypsinogen A + 820 maltose units of alpha-amylase per ml in saline.

Group E=1/10P “enzyme therapy, low concentrations” (14 mice): 750 BAEE units of trypsin + 3.1 BTEE units of alpha-chymotrypsinogen A + 820 maltose units of alpha-amylase per ml in saline.

Group Control “control group” (13 mice): saline

Tumor volumes were measured twice a week.

Proenzyme therapy of tumor bearing immunodeficient mice. We studied proenzyme therapy of female SCID mice bearing sarcoma S-180. We used proenzyme therapy based on mixture of trypsinogen (7500 BAEE units after activation/ml saline), alpha-chymotrypsinogen A (31
BTEE units after activation/ml saline) and alpha-amylase (820 maltose units/ml saline),
details are given in chapter “Results”.

*Determinaton of alpha-2 macroglobulin, contrapsin and alpha-1 antitrypsin in serum of
melanoma B16-F10 bearing C57BL/6 mice treated by proenzyme therapy.* The
experimental design was the same as the above mentioned proenzyme therapy of
melanoma. Details are given in the chapter “Results”. Levels of alpha-2 macroglobulin,
contrapsin and alpha-1 antitrypsin in pooled serum samples were determined using mass
spectrometry (MS). Samples were diluted 500 times using ammonium bicarbonate buffer
(c=100mmol/l, pH= 8.0, Sigma). We used enolase from yeast *Saccharomyces cerevisiae*
(Sigma) as an internal standard. Diluted samples were digested by trypsin (c= 100ng/ml,Sigma) for 12 hours at 37°C and analyzed using Q-ToF Premier mass
spectrometer (Waters Corporation, ESI-Q-ToF mass spectrometer coupled with a Nano
Acquity Liquid Chromatography device). The chromatographic column was filled by
hydrophobic compound (Bridged Ethyl Hydrid) with an eighteen carbon chain. The
chromatography analysis took 90 minutes. During the first 60 minutes we increased the
ratio of acetonitril to MilliQ Water (Millipore) from 3% to 60%. The flow was 400
nanoliters per minute.

We used the MS²E Identity method to obtain raw data, which was later processed by the PLGS
2.3 software (Waters). We used species-specific protein database containing mouse (*Mus
musculus*) entries from UniProt. The Expression Analysis function of the PLGS 2.3 software
was used for quantitative analysis. Absolute quantitative values of all analytes were obtained
on the basis of juxtaposition with concentration of murine albumin. All measurements were
tripllicated.
All of the experimental *in vivo* procedures were done in accordance with rules which are valid in EU and USA.

**Statistics.** Statistical analysis was performed using two-tailed Student’s t-test and the software STATISTICA VII, Survival Analysis (StatSoft, Inc., Tulsa, OK 74104, USA) at the significance level $2\alpha = 0.05$.

**RESULTS**

*Proenzyme therapy of sarcoma S-180 bearing mice.* Roughly 30% of the mice in each group failed to develop tumors which correspond with data published by others (Jones et al. 1939). Hence, the final number of mice in groups TG+CHG+A, TG+A, CHG+A and Control was 6, 7, 7 and 7 respectively. As shown in Fig. 1, the administration of combined trypsinogen, chymotrypsinogen and amylase significantly reduced tumor growth. Tumor volumes in this group averaged 40.3% those of the control group over a period of 14-61 days (mean of all measurements). Difference between group TG+CHG+A and Control increased over the course of the experiments and was significant at the significance level $\alpha = 0.05$ on days 49 and 61. Individual proenzymes showed substantially less effect than their mixture. The effect of trypsinogen and chymotrypsinogen was synergistic. In case of one mouse which survived for 115 days (group TG+CHG+A), tumor volume decreased from 4000 mm$^3$ on day 89 to 224 mm$^3$ on day 115 indicating cytoreduction consequent to proenzyme therapy. Fig. 2 shows survival in individual groups. In contrast to the control group, in which all mice died by day 85, the survival of mice in remaining groups (TG+CHG+A, TG+A, and CHG+A) on day 100 was 33%, 14% and 14% respectively. However, the differences between groups were not statistically significant.
In all three groups injected with proenzymes extensive surface lesions and tumor necrosis were observed. The surfaces of these tumors were usually concave. We observed no further change in groups TG+CHG+A, and TG+A, while group CHG+A exhibited a peripheral spread from the tumor edges. No surface lesions were found in the control group.

Remarkable differences were observed in tumor morphology. While most tumors in the control group had the shape of oblate spheroid, spreading in direction to the backbone, all tumors in group TG+CHG+A were spherical.

Upon dissection (immediately after death of each mouse), the biggest difference in size of extracted tumors was found between group TG+CHG+A (4026 ± 2514 mm³) and control group (6792 ± 2200 mm³). This difference was not statistically significant (P=0.06) due to different times of dissection. As shown previously, treated mice survived longer than controls and their tumors had more time to develop.

Histology did not reveal any significant differences between proenzyme treated and control group.

The experiment was repeated 5-times with similar results.

Proenzyme and enzyme therapy of melanoma B16-F10 bearing mice. C57BL/6 mice were injected with melanoma B16-F10 cells. Fig. 3 shows the development of tumors during experiment. The proenzyme-treated mice (group P) showed the largest reduction of tumor growth (to 45.2 % of control group volume) during the evaluated period of cancer treatment (14-32 day, mean of all measurements in this period). The difference between group P and the Control became statistically significant at the significance level alpha = 0.05 on the eighteenth day of experiment. Enzymes ten times less concentrated (group E=1/10P) reduced volume of tumors to 51.3% in comparison with the control (mean value in the same period).
We monitored the emergence of metastases (Table 1). Metastases were found in lungs only as melanoma B16-F10 is a lung metastases model. Group E=1/10P had the largest number of mice with metastases (58.3%). Group P had the lowest incidence (20%).

Fig. 4 shows the survival of mice. Mice in the group E=1/10P survived the longest, but the difference between this group and the control was not statistically significant. The difference between the group E=1/10P and E=PP (low versus high dose of enzymes) approached statistical significance (P = 0.058), indicating the need of optimization of dosage and precise dispensing in case of enzyme therapy.

The melanoma B16-F10 experiment (proenzyme therapy) was repeated six times with similar results.

One experiment (proenzyme therapy of melanoma B16-F10) was focused on measurement of total TGF-β in serum. Both proenzyme treated and control group contained 9 melanoma bearing mice each. Treatment started on day eleven after transplantation. We used i.p. application of both proenzymes and saline (control) as it proved to be more effective than i.m. application. Fig. 5 shows mean tumor volume in both groups. Proenzyme therapy strongly reduced tumor volumes, which was statistically significant at the end of the experiment. Fig. 6 shows total TGF-β determined in serum samples (each value is based on analysis of 3 serum samples). Significant reduction of TGF-β was detected at the end of the experiment.

In both sarcoma and melanoma experiments we observed time dependent changes of effectivity. Fig. 7 shows a typical course of effectivity of therapy dependent on its duration. At the beginning of the therapy both proenzymes and enzymes effectively hinder tumor growth. Then there is a period of time when the effect is reduced so much, that the tumors in treated group grow faster then in the control group. The effect of therapy stabilizes after that.
Proenzyme therapy of tumor bearing immunodeficient mice. To elucidate the mechanisms of proenzyme therapy, we compared its effect on BALB/c and SCID mice, both bearing sarcoma S-180. We used a well-tried dosage (7500 BAEE units of trypsinogen after activation + 31 BTEE units of alpha-chymotrypsinogen A after activation + 820 maltose units of alpha-amylase per ml in saline, daily i.m. application of 0.1 ml).

We started the therapy after tumors appeared (eleventh day after transplantation). Mice were divided into four groups: SCID + P (SCID mice with sarcoma, proenzyme treated, 10 mice), SCID (SCID mice with sarcoma, saline treated, control (10 mice), BALB/c +P (BALB/c mice with with sarcoma, proenzyme treated (10 mice), BALB/c (BALB/c mice with sarcoma, saline treated, control (9 mice). Proenzyme therapy revealed very strong effect on BALB/c mice with tumor volumes averaging 14.8% those of the control group over a period of 18-32 days (Fig. 8). Growth of sarcoma S-180 tumors on SCID mice was not influenced by proenzymes at all, tumors in proenzyme treated group grew even faster then in untreated control group.

Determination of alpha-2 macroglobulin, contrapsin and alpha-1 antitrypsin in serum of melanoma B16-F10 bearing C57BL/6 mice treated by proenzyme therapy. To elucidate the mechanism of the effect of proteases on tumors, we measured protease-activated alpha-2 macroglobulin levels during proenzyme therapy of transplanted melanoma B16-F10 versus untreated melanoma. Since other serum inhibitors (especially contrapsin and alpha-1 antitrypsin) compete in binding proteases, we determined their levels as well. Melanoma B16-F10 cells were transplanted to 28 female C57BL/6 mice. After 9 days tumor volumes were measured. Mice were randomly divided into two groups – treatment of group 1 started
immediately, group 2 served as a control. The treatment caused large reduction of tumor volume (to 42.1% versus control on average) during the evaluated period (15-29 day). The difference between treated and untreated mice was statistically significant at the significance level alpha = 0.05 on day 25 of the experiment (data not shown). Blood from tail vein was drawn in following intervals: before experiment, on day 17 of experiment (8 days of treatment), on day 29 of experiment (20 days of treatment). We performed MS analysis of the sera. Fig. 9 shows the dynamics of particular inhibitors. Mice treated by proenzyme therapy showed some statistically not significant increase of all inhibitors.

Table 2 summarizes all significant effects observed during whole study.

DISCUSSION

Proenzyme therapy significantly reduces growth of both sarcoma S-180 and of melanoma B16-F10. In case of sarcoma, the therapy prolongs survival time, causes tumors to retain a spherical tumor shape and hinders tumor penetration. In case of melanoma B16-F10 the prevalence of metastases was reduced.

In melanoma, a single concentration of proenzymes is optimal for both effective reduction of tumor growth and prevalence of metastases, while two different concentrations (by order of magnitude) are needed for active enzymes. This makes the proenzyme therapy more versatile.

We observed a strong synergy of trypsinogen and chymotrypsinogen. We optimized the amount of amylase. The supportive role of amylase in protease-based cancer treatment was proposed by Beard (1911) and confirmed by Novak and Trnka (2005). Based on our
experience from preliminary experiments, we used four times higher concentrations of amylase than used by Novak and Trnka (2005). This way we significantly reduced lethargy and decreased mobility of cured mice. What is the mechanism of action of amylase remains to be elucidated. One possibility is that amylase splits tumor glycogen (Rousset et al. 1980; Takahashi et al. 1999) released from tumors damaged by the therapy.

The concentration of trypsinogen used in experiments on both models correspond to study of Novak and Trnka (2005), who derived this concentration from reconstruction of original paper (Beard 1911). Although Beard did not write about chymotrypsin and chymotrypsinogen (both were discovered many years later), Novak and Trnka (2005) correctly realized, that Beard’s preparations had to contain chymotrypsinogen, but they did not use them in in vivo experiments.

Both proenzyme and especially enzyme therapy highlighted the importance of optimal therapeutic doses. The amount of enzymes and especially the means of their application are important. Enzyme preparations currently offered as supportive therapy (Wobe-Mugos E, Wobenzym® N) not only contain active proteases (misunderstanding of Beard’s papers), but their producers also recommend oral application. The question of absorbancy of proteases after oral administration is quite debatable. Although proteases are usually administered in large quantities and the daily dose reaches grams, some authors believe that they are not absorbed at all (Gewert et al. 2004), or only a small fraction of 0.002-0.0025% (Ziv et al. 1987), respectively 0.01 to 0.001% (Stastny et al. 2002) passes the intestine wall. There is no way to guarantee the exact dosage, which can be risky for patients, as too high or too low dosages might not work.

What is the mechanism of proenzyme therapy? We suppose that proenzymes as nonactive molecules migrate through blood circulation. They can pass into tumors on the basis of tumor vascular permeability. Their strong positive charge caused by high isoelectric point can
contribute to adhering on negatively charged tumor cells in solid tumors and metastases.

Rapidly growing and metastasizing tumors typically secrete large amounts of active proteases which may, in turn, cause local activation of the proenzymes (Novak and Trnka 2005). They comprise membrane-associated cathepsin B (Figarella et al. 1988; Kobayashi et al. 1993), tumor-derived trypsin (Koivunen et al. 1991; Nyberg et al. 2006), urokinase-type plasminogen activator (Uchima et al. 2003) and enterokinase (Miyata et al. 1999) or enterokinase-like enzymes (Nyberg et al. 2002). Autoactivation of trypsinogen by newly formed trypsin may also play a significant role in a ‘feed forward’ reaction (Kay and Kassell 1971). Trypsin as a key enzyme initiates chymotrypsinogen activation. In the case of sarcoma S-180 we even observed an effect of chymotrypsinogen alone, perhaps being activated by trypsin-like proteases previously described in this sarcoma (Chu et al. 1997).

The above steps allow active trypsin and chymotrypsin to accumulate in the site of tumor growth. Trypsin and chymotrypsin are trapped by alpha-2 macroglobulin. It leads to transformation of alpha-2 macroglobulin to its “fast” form connected with enhanced ability to bind cytokines (and eliminate them as resulting complexes are removed from blood). At this point the mode of action of proenzyme therapy is identical with mechanisms considered in the case of systemic enzyme therapy based on active enzymes, where the possibility of binding TGF-beta and IL-10 (cytokines, secreted by tumors to suppress immune system and establish tumor tolerance) is frequently discussed (Desser et al. 2001). We suppose that the main advantage of proenzyme therapy is activation of proenzymes mainly in tumor area. Therefore the impairment of immunotolerance is mainly local, not systemic, as in case of enzyme therapy.

The above mentioned mechanisms of (pro)enzyme therapy (binding of TGF-beta and IL-10) remain to be proven. Our experiment with SCID mice clearly shows that the effect of proenzymes (and expected high trypsin and chymotrypsin activities in place of tumor) is fully
dependent on the presence of completely developed acquired immunity. This experiment does
not directly prove proposed mechanisms, nevertheless all observations are in accordance with
this scheme. Binding and removing of TGF-β during proenzyme therapy of tumors was
directly supported by our experiment focused on measurement of TGF-β in murine sera.

Activating molecules (trypsin, chymotrypsin) and sufficient concentration of alpha-2
macroglobulin are both needed for formation of efficient “fast” alpha-2 macroglobulin. As we
observed, alpha-2 macroglobulin levels increased slowly during tumor growth and slightly
more rapidly in the course of proenzyme therapy of tumors. Sufficient and even increasing
concentration of alpha-2 macroglobulin enables function of proenzyme therapy during the
whole course of disease development. An important observation was, that two other inhibitors
(contrapsin and alpha-1 antitrypsin) did not show any dramatically high increase of
concentration. High levels of these inhibitors could compete for proteases with alpha-2
macroglobulin and therefore influence the development of necessary complexes.

We expect that the considered mechanism of inhibition of tumor growth by proenzyme
therapy (local activation of alpha-2 macroglobulin, augmentation of immune attack hindered
by TGF-beta) is valid for metastases as well. Since both the invading primary tumors and
metastases destroy surrounding tissues, the conditions for triggering the local activation of
proenzymes are fulfilled in both cases.

We can hardly explain the time fluctuations of proenzyme therapy. After first strong
effect of therapy (removing of TGF-beta and IL-10) it is indeed possible to expect that
activated alpha-2 macroglobulin will attack other cytokines important for development of
immune response. It contrasts with observations of Harthun et al. (1998), describing
preferential binding of TGF-beta and partial selectivity of this effect. Detailed understanding
of mechanism of proenzyme and enzyme effect on tumors and its changes in the course of
therapy will require further studies.
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Table 1. **Incidence of metastases in lungs of mice bearing melanoma B16-F10 and treated by proenzyme and enzyme therapy.**

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Incidence of metastases</th>
</tr>
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<tbody>
<tr>
<td>1 - proenzyme therapy (P)</td>
<td>20 %</td>
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<tr>
<td>2 – enzyme therapy (E=P)</td>
<td>25 %</td>
</tr>
<tr>
<td>3 – enzyme therapy, low concentrations (E=1/10P)</td>
<td>58.3 %</td>
</tr>
<tr>
<td>4 - control group (Control)</td>
<td>44.4 %</td>
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Table 2 **Summary of all significant effects observed during whole study.**

<table>
<thead>
<tr>
<th>Effects</th>
<th>Demonstrated</th>
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<tbody>
<tr>
<td>synergy between trypsinogen and chymotrypsinogen leads to significant reduction of tumor growth</td>
<td>Fig. 1</td>
</tr>
<tr>
<td>proenzyme therapy based on mixture of trypsinogen and chymotrypsinogen is effective in treatment of both sarcoma S-180 and melanoma B16-F10</td>
<td>Fig. 1, Fig. 3, Fig. 5, Fig. 8</td>
</tr>
<tr>
<td>proenzyme therapy reduces prevalence of metastases</td>
<td>Table 1</td>
</tr>
<tr>
<td>tumor bearing SCID mice did not respond on proenzyme therapy</td>
<td>Fig. 8</td>
</tr>
<tr>
<td>proenzyme therapy reduces level of serum TGF- β</td>
<td>Fig. 6</td>
</tr>
</tbody>
</table>
proenzyme therapy of melanoma B16-F10 bearing mice slightly enhances the serum levels of alpha-2 macroglobulin, contrapsin and alpha-1 antitrypsin

LEGEND OF FIGURES

Fig. 1. Growth of sarcoma S-180 tumors in mice treated with trypsinogen (TG), chymotrypsinogen (CHG), amylase (A) and combinations thereof. Mice were inoculated with 4 x 10^5 S-180 cells s.c., the treatment started on day 4 after tumor cell transplantation. Mice were injected daily (0.1 ml i.m.) with following solutions.

- **Group TG+CHG+A (9 mice):** 7500 BAEE units of trypsinogen (after activation) + 31 BTEE units of alpha-chymotrypsinogen A (after activation) + 820 maltose units of alpha-amylase per ml in saline.
- **Group TG+A (9):** 15000 BAEE units of trypsinogen (after activation) + 820 maltose units of alpha-amylase per ml in saline.
- **Group CHG+A (10):** 62 BTEE units of alpha-chymotrypsinogen A (after activation) + 820 maltose units of alpha-amylase per ml in saline.
- **Group Control (10):** saline.

* statistically significant as compared with control.
Fig. 2. Survival of mice with sarcoma S-180 treated with proenzyme therapies. The treatment was the same as in Fig. 1.

Fig. 3. Development melanoma B16-F10 tumors in mice treated by proenzyme and enzyme therapy. Mice were inoculated with $4 \times 10^5$ B16-F10 cells s.c., the treatment started on day 11 after tumor cell transplantation. Mice were injected daily (0.1 ml i.m.) with following solutions.
Group P “proenzyme therapy” (14 mice): 7500 BAEE units of trypsinogen (after activation) + 31 BTEE units of alpha-chymotrypsinogen A (after activation) + 820 maltose units of alpha-amylase per ml in saline.

Group E=P “enzyme therapy” (14 mice): 7500 BAEE units of trypsin + 31 BTEE units of alpha-chymotrypsinogen A + 820 maltose units of alpha-amylase per ml in saline.

Group E=1/10P “enzyme therapy, low concentrations” (14 mice): 750 BAEE units of trypsin + 3.1 BTEE units of alpha-chymotrypsinogen A + 820 maltose units of alpha-amylase per ml in saline.

Group Control (13 mice): saline

Symbols as in Fig. 1.

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**Fig. 4.** Survival of mice with melanoma B16-F10 treated by proenzyme and enzyme therapy. The treatment was the same as in Fig. 3.
Fig. 5. Proenzyme therapy of melanoma B16-F10 bearing mice (experiment focused on measurement of TGF-β). Mice were inoculated with $4 \times 10^5$ B16-F10 cells s.c., the treatment started on day 11 after tumor cell transplantation. Mice were injected daily (0.1 ml i.p.) with following solutions. Group P (9 mice): 7500 BAEE units of trypsinogen (after activation) + 31 BTEE units of alpha-chymotrypsinogen A (after activation) + 820 maltose units of alpha-amylase per ml in saline. Group Control (9 mice): saline.

Symbols as in Fig. 1.

Fig. 6. Proenzyme therapy of melanoma B16-F10 bearing mice – determination of total serum TGF-β. The treatment was the same as in Fig. 5. TGF-β was determined by ELISA in three serum samples per time point from each group.
Fig. 7. **Typical course of proenzyme and enzyme therapy of melanoma B16-F10.** Group P means proenzyme therapy based on mixture of trypsinogen and chymotrypsinogen with supportive amylase in basic dose (trypsinogen, 7500 BAEE units after activation + alpha-chymotrypsinogen A, 31 BTEE units after activation + alpha-amylase, 820 maltose units per ml in saline). Group E=1/10P means enzyme therapy based on the use of active proteases in ten times lower molar concentration than that of proenzymes (750 BAEE units of trypsin + 3.1 BTEE units of alpha-chymotrypsinogen A + 820 maltose units of alpha-amylase per ml in saline. Tumor volumes in treated groups are expressed as a percentage of tumor volumes in control untreated group in particular time points.
Fig. 8. **Proenzyme therapy of SCID and BALB/c mice bearing sarcoma S-180.**

Mice were inoculated with \(4 \times 10^5\) S-180 cells s.c., the treatment started on day 11 after tumor cell transplantation. Mice were injected daily (0.1 ml i.m.) with following solutions. Group SCID+P (10 SCID mice) and group BALB/c+P (10 BALB/c mice): 7500 BAEE units of trypsinogen (after activation) + 31 BTEE units of alpha-chymotrypsinogen A (after activation) + 820 maltose units of alpha-amylase per ml in saline.

Group SCID (10 SCID mice) and BALB/c (9 BALB/c mice): saline.

* statistically significant as compared with control (BALB/c)
alpha-2 macroglobulin

days

Naive ■ Proenzymes □ Control

contrapsin

days

Naive ■ Proenzymes □ Control

alpha-1 antitrypsin

days

Naive ■ Proenzymes □ Control
Fig. 9. in Serum levels of alpha-2 macroglobulin (subunit – Mw 165 722), contrapsin
(Mw 45 969) and alpha-1 antitrypsin (Mw 46 850) melanoma B16-F10 bearing C57BL/6 mice

treated with proenzyme therapy. Therapy (as in Fig. 8) started on day 9 after tumor cell
transplantation. All protease inhibitors were determined by MS. Treated and control groups contained
14 mice each.