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### Direct ELISA Method for the Specific Determination of Prothymosin Alpha in Human Specimens

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**DIRECT ELISA METHOD FOR THE SPECIFIC DETERMINATION OF  
PROTHYMOSIN ALPHA IN HUMAN SPECIMENS**

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**ABSTRACT**

An enzyme linked immunosorbent assay, specific for prothymosin alpha (ProT $\alpha$ ) was developed using an antibody against the synthetic C-terminal peptide ProT $\alpha$ [101-109] and isolated bovine ProT $\alpha$  for the preparation of standard solutions and immunoplates. Due to the antibody used, the ELISA developed was capable of fully discriminating between ProT $\alpha$ , the naturally occurring and partially homologous peptide parathymosin alpha (ParaT $\alpha$ ) and the peptide thymosin  $\alpha_1$  (T $\alpha_1$ ), whose sequence is identical to the [1-28] sequence of ProT $\alpha$ , and its *in vivo* occurrence is under question. Moreover, due to its improved sensitivity, the ELISA was capable of directly determining ProT $\alpha$  concentration in human serum and tissue extracts, without any pretreatment of the samples. ProT $\alpha$  levels were directly measured in sera obtained from 48 apparently healthy individuals and 27 patients with diagnosed breast cancer and found to range from 0.67 to 2.34  $\mu\text{g/ml}$  (mean value  $1.27 \pm 0.49 \mu\text{g/ml}$ ) and from 0.47 to 1.74  $\mu\text{g/ml}$  (mean value  $1.02 \pm 0.29 \mu\text{g/ml}$ ), respectively. ProT $\alpha$  levels were also measured in four breast tumor and adjacent normal breast tissue extracts and found to be elevated in the tumor extracts.

(KEY WORDS: prothymosin  $\alpha$ , breast cancer, immunoassay)

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## INTRODUCTION

Human ProT $\alpha$  is an acidic protein (pI = 3.55), consisting of 109 amino acid residues ( $M_r$  = 12.6 kDa), which is probably involved in both extracellular and intracellular biological functions. ProT $\alpha$  extracellular biological role, studied shortly after the protein isolation and characterization, was found to be related to cell immunity: thus, ProT $\alpha$  appears to be effective in immuno-protection of mice against opportunistic infections (1). More recently, there has been increasing interest in ProT $\alpha$  intracellular role as well. As it has been shown, ProT $\alpha$  might be a nuclear protein containing a functional nuclear localization signal in the C-terminus (2), which was identified as the sequence 101-104 (KKQK). Through this sequence the protein is considered to be transported into the cell nucleus (3), where it binds to histone H1, as *in vitro* experiments have shown (4). Recent publications provide evidence that ProT $\alpha$  may be involved in normal and abnormal cell proliferation mechanisms. Thus, ProT $\alpha$  RNA levels were increased when several cell types were stimulated to proliferate (5-7). In addition, ProT $\alpha$  antisense oligomers were shown to inhibit myeloma cell division (8), while activation of the transcription of the protooncogene *myc* increased the transcription of the ProT $\alpha$  gene, even in the absence of protein synthesis (9). According to accumulated research findings, tumor ProT $\alpha$  levels can be considered a proliferation index of human breast cancer and might be used for identifying patients at high risk for distant metastasis (10).

ProT $\alpha$  is closely related to the 28 amino acid residue - peptide T $\alpha_1$ , first isolated from thymosin fraction-5 (11), the sequence of which is identical to the first twenty

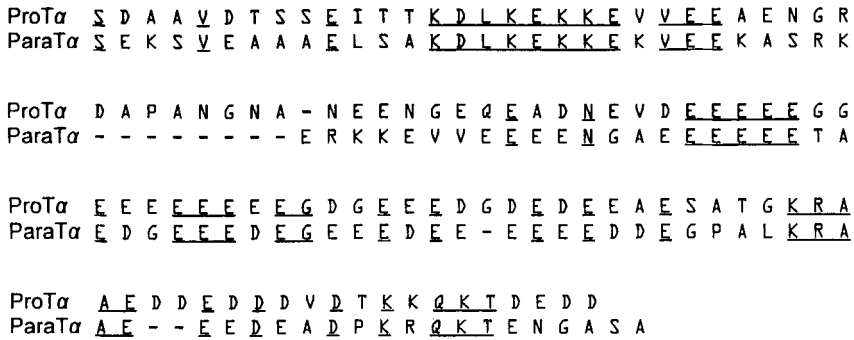


Figure 1. Primary structure of ProTα and ParaTα. The [1-28] sequence of ProTα represents Tα<sub>1</sub>. Common amino acid residues are underlined.

eight N-terminal amino acids of ProTα. According to some researchers, Tα<sub>1</sub> is not a natural peptide, but rather the product of proteolytic cleavage of ProTα during tissue extraction procedures (12). However, other investigators suggest that both peptides occur in intact tissues *in vivo* (13), the issue being still obscure. The naturally occurring polypeptide ParaTα also presents sequence similarities (45 %) with ProTα (Figure 1).

Tumor ProTα levels that have been so far reported in the literature were determined using mainly radioimmunoassays (RIAs) or enzyme-linked immunosorbent assays (ELISAs). The antisera used in the above immunoassays were developed mainly against Tα<sub>1</sub>, measuring therefore both ProTα and Tα<sub>1</sub> peptides. In a recent publication (14), the authors calculate ProTα concentrations in human tumor extracts by measuring Tα<sub>1</sub> concentration assuming an one-to-one correspondence between ProTα and Tα<sub>1</sub>, but the possibility of the *in vivo* presence of Tα<sub>1</sub> has not yet been excluded. The only exception to the use of antisera against

the amino terminus of ProT $\alpha$  is the antiserum used in one RIA method, which had been developed against the C-terminal fragment [90-109] of ProT $\alpha$  (15). Moreover, all ProT $\alpha$  immunoassays so far developed are not capable of analyzing untreated (i.e. non concentrated) serum samples, mainly due to their lack of sensitivity, although this may prove to be very useful. In this paper we present a specific and sensitive ProT $\alpha$  - ELISA method, which is based on an antiserum raised against the specially selected C-terminal epitopic fragment [101-109] of ProT $\alpha$ . The ELISA method developed was capable of directly determining ProT $\alpha$  concentrations in untreated human serum samples and tissue extracts.

## **MATERIALS AND METHODS**

### **Materials**

Prothymosin  $\alpha$ , isolated from bovine tissues by means of a method previously described for rat tissues (12), was a generous gift of Thymoorgan GmbH Pharmazie & Co (Vienenburg, Germany). Analytical grade reagents were purchased from Merck or Sigma, except as otherwise indicated. *Fmoc* protected amino acids were obtained from CBL-Co (Patras, Greece). The ultra pure water used was produced by Barnstead Easy Pure apparatus. HPLC grade acetonitrile was obtained from Lab-Scan. Complete Freund's Adjuvant was a product of Difco. ELISA plates were purchased from Costar. Conformational/antigenic predictions were performed using the PC/Gene release 6.85 package from IntelliGenetics Inc. Serum samples from apparently healthy male and female individuals aged between 18-60 years and patients with diagnosed breast cancer of

several types and stages, were kindly provided by Dr. A. Stathaki-Ferderigou, "Agios Savas" Hellenic Anticancer Hospital, and kept at  $-35^{\circ}\text{C}$  until used.

Tumor and adjacent normal tissue samples, kindly provided by Dr. N. Apostolikas, were obtained from 4 female, middle aged patients with primary breast cancer undergoing definitive surgery at the "Agios Savas" Hellenic Anticancer Hospital. All tumors were ductal carcinomas, about 2 cm in diameter.

#### Preparation of Tumor and Normal Tissue Extracts

Tumor and normal tissue samples were homogenized with a Janke & Kunkel Ultra Turrax T25 homogenizer at room temperature in phosphate buffered saline (PBS) pH 7.4 and PBS pH 7.4 containing 5  $\mu\text{g}/\text{ml}$  aprotinin, for 3 min. The homogenates were centrifuged at 14000 x g for 15 min at  $4^{\circ}\text{C}$  and the supernatants centrifuged for another 15 min at 14000 x g. The final supernatants were aliquoted and stored at  $-35^{\circ}\text{C}$ . The total protein content of the supernatants was determined by the Lowry assay.

#### Synthesis of Prothymosin $\alpha$ Fragments

Prothymosin  $\alpha$  fragments ProTa[87-109] and ProTa[101-109] were synthesized manually by the *Fmoc*-solid-phase strategy on a para-cyano-trityl resin as previously reported for the synthesis of the 43 amino acid residue - peptide thymosin  $\beta$ -10 (16).

The synthetic peptides were purified by semi-preparative RP-HPLC on a Nucleosil C-18 column (250x10 mm i.d., 7  $\mu\text{m}$  particle size, Macherey-Nagel) using 0.05% TFA/acetonitrile gradient in 0.05% trifluoroacetic acid (TFA) in water as eluent. Peptide purity was tested on a LiChrospher RP-C18 analytical

column (250x4.6 mm i.d., 5  $\mu$ m particle size, Merck) using the above described elution system. HPLC analysis and purification were performed on a Waters liquid chromatography apparatus, equipped with pump model 600 and an absorbance UV detector model 484 set at 220 nm. Amino acid analysis was performed using the Pico-Tag method (Waters).

#### Preparation of Immunogens and Polyclonal Antisera

For immunogen preparation, the synthetic peptides as well as isolated ProT $\alpha$  were coupled to keyhole limpet hemocyanin (KLH) through their amino groups by the glutaraldehyde method (17).

New Zealand white rabbits were intradermally injected with 20  $\mu$ g of the corresponding synthetic peptide or 75  $\mu$ g of the isolated ProT $\alpha$ , in the KLH-conjugated form emulsified in Complete Freund's Adjuvant, according to the method of Vaitukaitis (18). The animals were boosted initially after six weeks and subsequently every four weeks. Blood was collected two weeks after each booster injection. The antisera were obtained with low speed centrifugation and stored at -35  $^{\circ}$ C.

#### Buffers and Solutions

*Coating buffer:* citrate buffer 0.15 M pH 5.0

*Washing buffer:* PBS pH 7.4 (15 mM KH<sub>2</sub>PO<sub>4</sub>, 8mM Na<sub>2</sub>HPO<sub>4</sub>.2H<sub>2</sub>O, 150 mM NaCl, 2.7 mM KCl) containing 0.05% v/v Tween 20.

*Dilution buffer:* 0.2% w/v BSA (bovine serum albumin) in washing buffer

*Substrate solution pH 4.5:* 0.1 M Na<sub>2</sub>HPO<sub>4</sub>.2H<sub>2</sub>O, 0.1 M citric acid, 0.003% v/v

H<sub>2</sub>O<sub>2</sub>, 1% w/v 2,2'-azinobis-(3-ethyl-2,3-dihydrobenzothiazole-6-sulfonic acid)diammonium salt (ABTS).

#### Coating of the ELISA Microwells

The ELISA microwells were coated with 200 µl/well ProTα solution (0.1 µg/ml) in coating buffer and incubated overnight at 37 °C. The coating solution was then discarded and the microwells were rinsed twice with PBS pH 7.4. Afterwards, the microwells were incubated (200 µl/well) with a 2% BSA solution in dilution buffer (blocking solution) for 1 h at 37 °C. After incubation, the blocking solution was discarded and the microwells were rinsed three times with washing buffer. The so prepared ProTα coated microwells were then ready for use in titer determination, displacement curve or ELISA measurement experiments.

#### Determination of Primary Antiserum Titer

Serial dilutions of primary antiserum in dilution buffer (200 µl/well) were added to the ProTα coated microwells and incubated for 2 h at 37 °C. Goat anti-rabbit IgG conjugated to horseradish peroxidase, at 1:3,000 dilution in dilution buffer (200 µl/well), were added and incubated for 2 h at 37 °C. Each incubation step was followed by three rinses with washing buffer. Colour was developed by addition of 200 µl/well ABTS solution and optical absorbance measured after 30 min at 405 nm using an automatic microtiter plate reader (Dynatech MR5000). As a negative control, serial dilutions of non-immune rabbit serum and anti-KLH rabbit antiserum were added instead of primary antiserum. Additionally, cross-reactivity of the primary antisera against Tα<sub>1</sub> or ParaTα was tested using Tα<sub>1</sub> or ParaTα coated microwells, respectively.



### Displacement Curves

Equal volumes of ProT $\alpha$  standard solutions (0.01, 0.03, 0.1, 0.3, 1, 3, 10  $\mu\text{g/ml}$  ProT $\alpha$  in dilution buffer) and primary antiserum solutions in dilution buffer were vortexed in polystyrene tubes and then immediately pipetted into the ProT $\alpha$  coated microwells (200  $\mu\text{l/well}$ ) and incubated for 2 h at 37  $^{\circ}\text{C}$ . The following steps were performed as above described for antiserum titer.

### ELISA Measurement

Equal volumes of ProT $\alpha$  standard solutions (0.01, 0.03, 0.1, 0.3, 1, 3, 10  $\mu\text{g/ml}$  ProT $\alpha$  in dilution buffer) or serum samples or tissue supernatants and antiserum against ProT $\alpha$ [101-109], diluted 1:3,000 in dilution buffer, were vortexed in polystyrene tubes and then immediately pipetted into the ProT $\alpha$  coated microwells (200  $\mu\text{l/well}$ ) and incubated for 2 h at 37  $^{\circ}\text{C}$ . After discarding and rinsing as above, goat anti-rabbit IgG conjugated to horseradish peroxidase, at 1:3,000 dilution in dilution buffer (200  $\mu\text{l/well}$ ), was added and incubated for 2 h at 37  $^{\circ}\text{C}$ , followed by discarding and rinsing. Colour was developed by adding 200  $\mu\text{l/well}$  ABTS solution and the optical absorbance was measured after 30 min at 405 nm.

## RESULTS

### Coating of the ELISA Microwells

For optimizing the coating conditions, three different coating buffers were tested: PBS pH 7.4, citrate buffer 0.15 M pH 5.0, and carbonate-bicarbonate buffer 0.05 M pH 9.2. Also, different ProT $\alpha$  coating concentrations were tested. After adding the ProT $\alpha$  coating solution, the ELISA microwells were dried

overnight at 50 °C or incubated overnight at 37 °C. Best results were obtained with citrate buffer 0.15 M pH 5.0, ProTα coating concentration equal to 0.1 µg/ml, and overnight incubation at 37 °C.

#### Evaluation of the Antisera

Several bleedings of three different primary antisera, namely antisera against ProTα, ProTα[87-109] or ProTα[101-109] (Ab-ProTα, Ab-ProTα[87-109], or Ab-ProTα[101-109], respectively), were tested for their titer (Figure 2). The highest titer values obtained for each antibody tested were: Ab-ProTα 1:4,000 (first bleeding), Ab-ProTα[87-109] 1:1,000 (fourth bleeding), Ab-ProTα[101-109] 1:6,000 (sixth bleeding).

#### Displacement Curves

The displacement curves obtained with the three primary antisera tested are shown in Figure 3. Ab-ProTα and Ab-ProTα[101-109] gave displacement curves with the broadest useful range, from 10 ng/ml to 10 µg/ml.

#### ELISA Method

The inter-assay CV determined from five standard curves run in duplicate over a period of 10 weeks was 14%, while the intra-assay CV determined from a pooled serum sample measured ten times in duplicate was 3%. The absorbance value of the zero standard was  $1.18 \pm 0.04$  AU (absorbance units). The assay detection limit was estimated to be less than 10 ng/ml, corresponding to 0.072 AU, defined as 2 SD of the zero standard absorbance values from five standard curves. The useful range of the standard curve was 10 ng/ml to 10 µg/ml. The non-specific binding was calculated from the values obtained from ProTα coated wells incubated

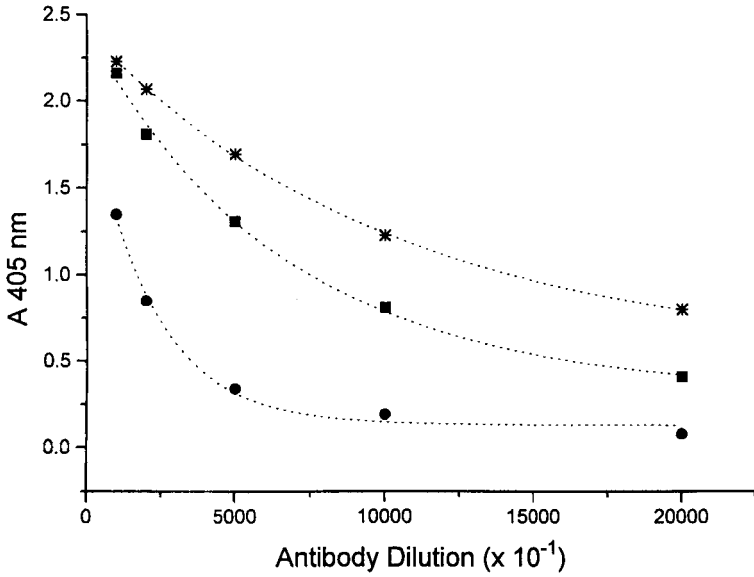


Figure 2. Titer curves obtained with the antisera tested (■ : antiserum against ProT $\alpha$ , ● : antiserum against ProT $\alpha$ [87-109], \* : antiserum against ProT $\alpha$ [101-109]).

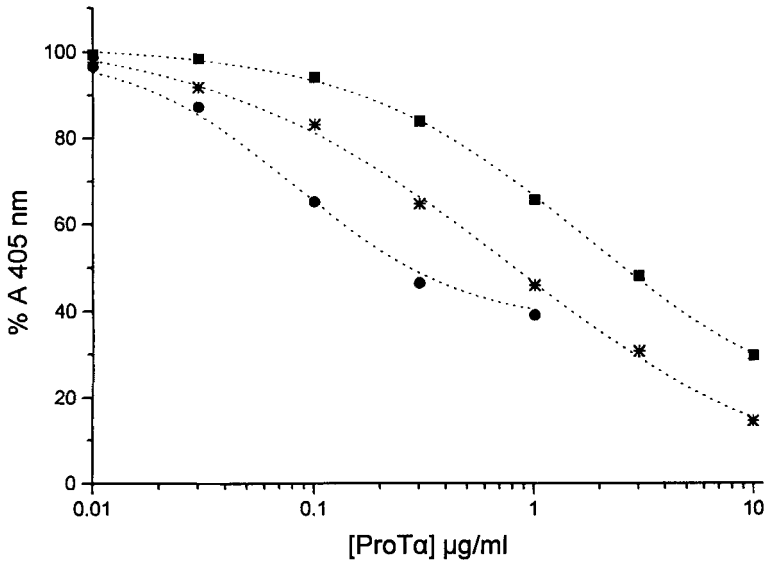


Figure 3. ELISA-displacement curves obtained with the antisera tested (■ : antiserum against ProT $\alpha$ , ● : antiserum against ProT $\alpha$ [87-109], \* : antiserum against ProT $\alpha$ [101-109]).

directly with labelled secondary antibody, with uncoated wells incubated with both primary and secondary antibodies and finally with ProT $\alpha$  coated microwells incubated with non-immune rabbit serum instead of primary antibody. In all cases, the non-specific binding value did not exceed 3%.

Analytical recovery was tested by addition of 0.1 and 1  $\mu\text{g/ml}$  and was defined as  $\mu\text{g}$  recovered divided by  $\mu\text{g}$  expected times 100. It was found 110% (0.1  $\mu\text{g}$  added) and 101% (1  $\mu\text{g}$  added) respectively. Dilution experiment of one serum sample of known concentration was also performed. The sample was measured diluted 1:2, 1:4 and 1:8. Recovery in this case was defined as concentration measured divided by concentration expected times 100, and found 86% (1:2 dilution), 94% (1:4 dilution) and 106% (1:8 dilution) respectively.

The calibration curve plotted as the percentage of absorbance measured ( $A/A_0 \times 100$ ) against serial concentrations of ProT $\alpha$  on a logarithmic scale is linear in the concentration range 0.1-10  $\mu\text{g/ml}$ . The calibration curve is shown in Figure 4. Linear regression analysis of the calibration curve in this range, gave an intercept of 150, and a slope of -34;  $r^2$  was 0.997, SE was 1.5 and P was 0.00005.

#### Prothymosin $\alpha$ Levels in Human Serum and Tissue Samples

Serum samples collected from 48 apparently healthy individuals and 27 patients with diagnosed breast cancer were analyzed without any pretreatment. The values determined ranged from 0.67 to 2.34  $\mu\text{g/ml}$  (mean value  $1.27 \pm 0.49 \mu\text{g/ml}$ ) and from 0.47 to 1.74  $\mu\text{g/ml}$  (mean value  $1.02 \pm 0.29 \mu\text{g/ml}$ ), respectively (Figure 5).

ProT $\alpha$  levels in all tumor samples examined were greater than ProT $\alpha$  levels found in adjacent normal breast tissue of the same patient. The results (ng

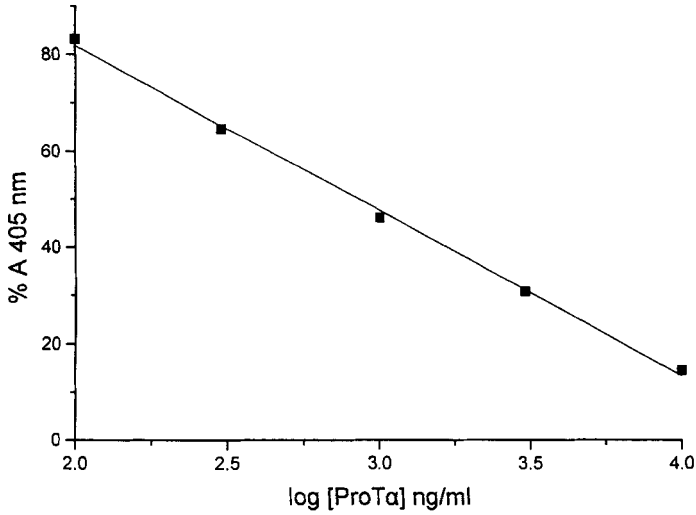


Figure 4. ProT $\alpha$  calibration curve obtained with the antiserum against ProT $\alpha$ [101-109]

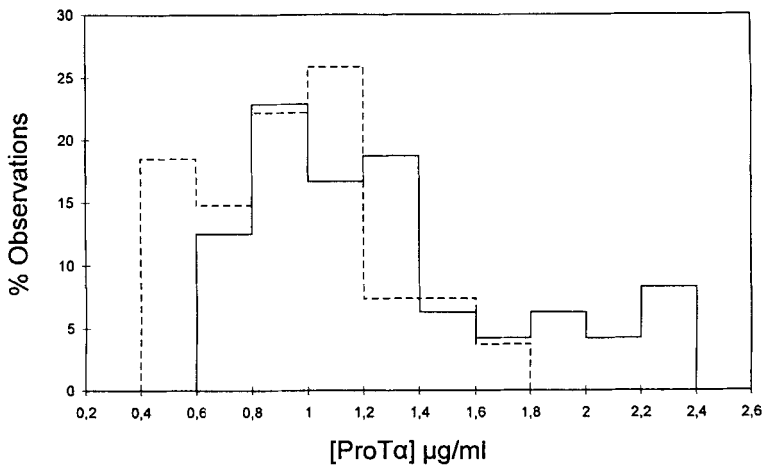


Figure 5. Serum ProT $\alpha$  concentrations in healthy individuals (—) and patients with primary breast cancer (---)

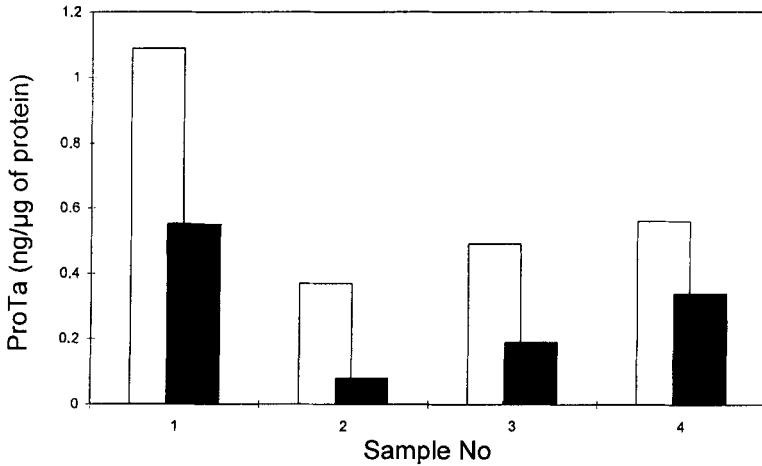


Figure 6. ProTa concentrations in breast tumor (□) and adjacent normal tissue extracts (■)

ProTa/μg of protein), are shown in Figure 6. No difference was observed between measurements of tissue samples homogenized in PBS or PBS containing aprotinin.

### DISCUSSION

According to recent findings, increased ProTa levels in human tissues may be associated with the development of certain tumors. In order to facilitate further relevant research, we have developed and present here a specific and sensitive ELISA method for determining ProTa in human specimens. We also report preliminary data concerning ProTa concentration in untreated serum samples of healthy individuals and diagnosed breast cancer patients as well as in breast tumor and normal breast tissue extracts.

The ELISA developed is specific for ProTa, since it is based on an antiserum raised against the C-terminal fragment [101-109] of ProTa, which presents no

sequence similarities with  $T\alpha_1$  or Para $T\alpha$  (Figure 1). Moreover, a computer-assisted search into the protein databank of the European Molecular Biology Laboratory, using the BLITS server, showed that the amino sequence of this peptide is not present in any other known human protein. The C-terminal fragment [101-109] of Pro $T\alpha$  was selected as the antigen of choice because of its amino acid sequence, which is the basis of the corresponding antiserum specificity, and also because of its putative epitopic nature. The latter was revealed after theoretically studying the structure of the Pro $T\alpha$  molecule.

In order to derive structure-antigenicity relationships we have applied secondary structure prediction by the well established methods of Chou and Fasman (19) and Gascuel and Goldmard (20). The analysis suggested that Pro $T\alpha$  adopts an extended structure composed by helical segments including residues 12-26, 44-66, 72-82, 86-96 with random coil segments separating these fragments. These findings are in agreement with those previously reported (21,22). The amino-acid sequence of Pro $T\alpha$  was also analyzed for identifying regions with high local average hydrophilicity by the method of Hopp and Woods (23) and high indices of flexibility by the method of Karplus and Schulz (24). As shown in Figure 7, the carboxy-terminus of Pro $T\alpha$  is predicted to contain a major antigenic determinant between the sequence 88-108, which shows the highest hydrophilicity and flexibility indices.

The carboxy-terminal fragments [87-109] and [101-109] of Pro $T\alpha$ , both ending at a lysine residue at the N-terminus, were synthesized for antiserum development. For the immunogen preparation, the peptide fragments synthesized were coupled

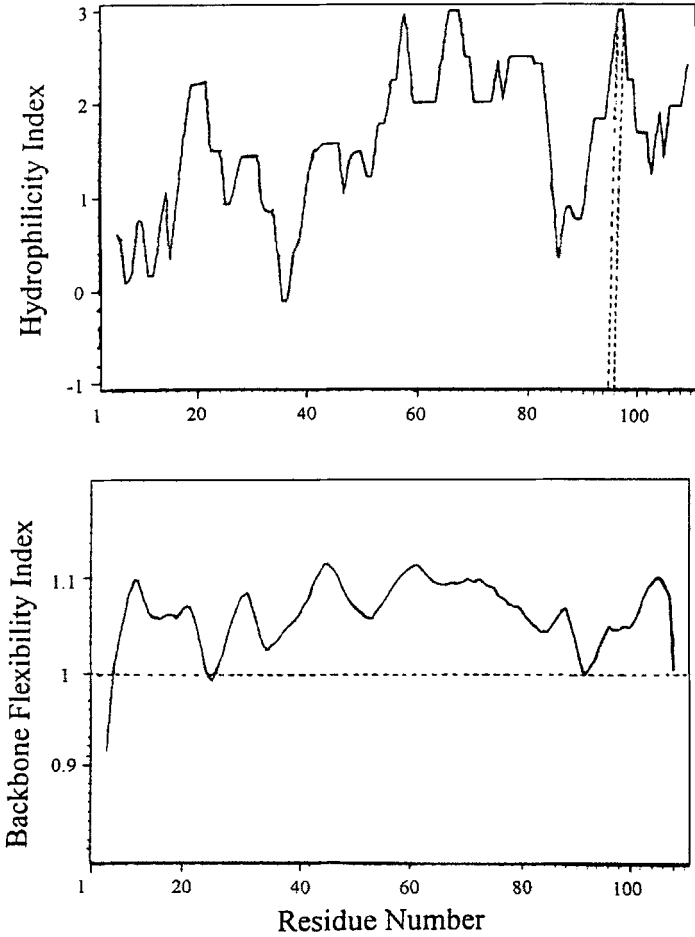


Figure 7. Theoretical data concerning hydrophilicity profile (upper) and backbone flexibility (lower) of human ProT $\alpha$ .



to KLH, through their lysine  $\epsilon$ -NH<sub>2</sub> and  $\alpha$ -NH<sub>2</sub> groups, following the glutaraldehyde method. The whole molecule of natural ProT $\alpha$ , isolated from bovine tissues, was also coupled to KLH and used for raising antiserum, as the control immunogen. The antisera raised against ProT $\alpha$ , ProT $\alpha$ [87-109], or ProT $\alpha$ [101-109] were all able to recognize natural ProT $\alpha$ , as shown with titer and displacement curves, while antisera raised against KLH or non-immune rabbit sera (negative controls) were not. Due to the corresponding aminoacid sequences, the antisera raised against the C-terminal fragments [87-109] and [101-109] of ProT $\alpha$  were considered more specific than that raised against intact ProT $\alpha$ . Between the former ones, the antiserum against the fragment ProT $\alpha$ [101-109] was finally selected for developing an ELISA method for ProT $\alpha$ , since it showed higher titer (1:6,000) and broader working range (Figure 2, 3).

Due to the antiserum used, the ELISA method developed was capable of fully discriminating between ProT $\alpha$ , ParaT $\alpha$  and T $\alpha$ <sub>1</sub> as shown in the titer curves of anti-ProT $\alpha$ [101-109] against ProT $\alpha$ , ParaT $\alpha$  and T $\alpha$ <sub>1</sub> (Figure 8) and in the displacement curve with ParaT $\alpha$  or T $\alpha$ <sub>1</sub> standards (data not shown). To our knowledge, the only immunoassay described in the literature so far that uses antibodies specific for ProT $\alpha$  is the radioimmunoassay which is based on antibodies raised against the C-terminal fragment [90-109] of ProT $\alpha$  (15). Compared with this specific radioimmunoassay, the ELISA here described is more sensitive (detection limit less than 10 ng/ml) and can therefore be used for the direct analysis of unconcentrated human serum samples, as explained below. The better sensitivity achieved might be due to: i) the avoidance of directly radiolabelling a ProT $\alpha$  tyrosine-derivative

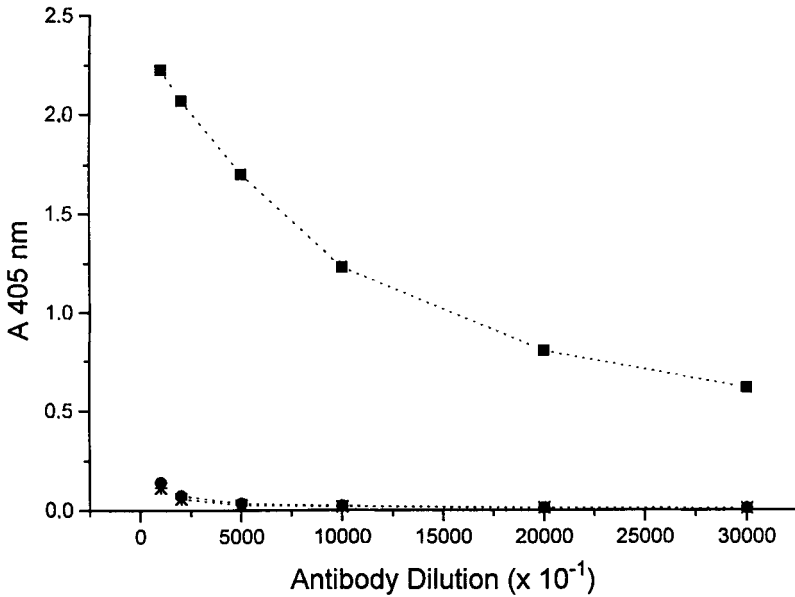


Figure 8. Crossreactivity of the antiserum against ProT $\alpha$ [101-109] with ProT $\alpha$  (■), ParaT $\alpha$  (●) and T $\alpha_1$  (\*).

fragment and using it as a radiotracer in the immunoassay system, which seems to deteriorate the immunochemical reaction characteristics; ii) the apparently higher  $K_{aff}$  of the antibody used, which might be attributed to the epitopic fragment selected for antibody raising.

The improved sensitivity characteristics of the ELISA method developed resulted in using highly diluted antiserum (1:6,000) in the assay measurements, with the consequent, well established advantages (avoidance of matrix-effects, lack of non-specific non-specificity, etc.). To the contrary, in the RIA method previously mentioned, the antiserum was used in a dilution even less than 1:500.

Due to its characteristics, the ELISA method developed was capable of measuring directly ProT $\alpha$  in human serum samples, without any pretreatment, thus providing a valuable tool for investigating the diagnostic importance of serum ProT $\alpha$  levels. Serum is an "ideal" clinical specimen, since it can be easily and reproducibly obtained. Till now, however, only few immunoassays have been described for ProT $\alpha$  serum measurements and they usually include a rather tedious and time consuming pretreatment step, e.g. through Sep-Pak cartridges and Speed-Vac procedure, so as to concentrate the samples, which can also affect the accuracy and reproducibility of the measurement (25). For this reason mainly, the diagnostic significance of serum ProT $\alpha$  levels in human cancerous diseases has not yet been thoroughly investigated.

ProT $\alpha$  levels were directly measured in serum samples obtained from 48 apparently healthy individuals using the ELISA method developed. According to the results obtained, serum ProT $\alpha$  concentrations ranged between 0.67 and 2.34  $\mu\text{g/ml}$  (mean value =  $1.27 \pm 0.49 \mu\text{g/ml}$ ). Serum ProT $\alpha$  levels were also measured in 27 patients with breast cancer. The values obtained ranged from 0.47 to 1.74  $\mu\text{g/ml}$  (mean value =  $1.02 \pm 0.29 \mu\text{g/ml}$ ).

In order to validate the reliability of the method, ProT $\alpha$  levels were also measured in breast tumor and adjacent normal tissue extracts. ProT $\alpha$  levels in all tumor samples examined were found elevated compared to those of the adjacent normal tissue, as it has been previously reported (10,26). No difference was observed in measurements between tissue samples homogenized in PBS and PBS containing aprotinin, which is a protease inhibitor. This observation suggests that

possible cleavage of ProT $\alpha$  by tissue proteolytic enzymes during extraction, does not affect the method.

In conclusion, a specific, sensitive and easy-to-perform ELISA method was developed, able to determine ProT $\alpha$  levels in clinically important human specimens. This ELISA was used for determining ProT $\alpha$  levels in sera obtained from healthy individuals and patients with diagnosed breast cancer. It was also applied to preliminary studies involving breast tumor and normal breast tissue extracts.

According to the relevant results there seems to be no elevation in serum ProT $\alpha$  levels in breast cancer patients compared to those found in the control individuals. Thus, although ProT $\alpha$  levels in breast tumors were found to be elevated using either immunoassays or immunohistochemical techniques, as previously reported by our group and other investigators (10, 26-28), no such difference was observed comparing serum ProT $\alpha$  levels of breast cancer patients and those of healthy persons. This may be an evidence for the discrete intracellular and extracellular roles that ProT $\alpha$  may play in human. However, more detailed clinical studies involving groups of patients with strictly defined characteristics are necessary, in order to further elucidate whether there is any association between serum ProT $\alpha$  levels and cancerous diseases.

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