IDENTITY OF PHOSPHOTRANSFERASE AND PHOSPHOMONOESTERASE OF HUMAN PROSTATE AND OF SERA FROM PATIENTS WITH PROSTATIC CANCER*

by

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INTRODUCTION

Recent work[1-5] has demonstrated the phosphotransferase properties of several phosphatase preparations. In at least two papers[5,6] it is stated, or implied, that the hydrolytic and transferring properties are both functions of the same enzyme. This assumption is based on the observation that in partially purified enzyme preparations the two functions are present in the same proportions. It has not been shown that the phosphomonoesterase appearing in human serum in advanced cancer of the prostate is identical with that of the normal prostate.

The results reported here demonstrate that the acid phosphatase of the prostate is identical with the transferase in physical characteristics, and that the prostatic and serum enzyme are physically and functionally identical.

METHODS AND MATERIALS

Diffusion measurements

A modification of FRICK's[7] technique for the measurement of diffusion constants of small molecules was used to allow crude prostatic extract[8] to diffuse into an agar cylinder. The system consisted of a museum jar containing the extract, fitted with a series of agar tubes (cylinders) so that one end of each was immersed in the prostate extract. The immersed ends were cut flat, and there was agar-extract contact. The system was gently stirred. The temperature of the museum jar and its contents were maintained at 1° ± 0.5° C by being almost completely immersed in an ice-water mixture. The pH (5.0) of the system (the extract and 1.5% agar gel) was maintained by a 0.2 *N* acetate buffer. The size of the museum jar was chosen so that there were no appreciable changes in enzyme concentration in the extract even though some activity diffused in the agar during the course of the experiment. Agar tubes were withdrawn at various times, the agar extruded and sliced with a razor edge, perpendicular to the long axis of the cylinder, into thin sections. The slices were weighed on tared wax paper and immersed with the wax paper in 0.2 *N* acetate buffer at pH 5.0 containing 0.02% egg albumin. The solutions were refrigerated and the contents of the slices extracted for 5 days or longer. The resulting solutions were assayed for phosphatase[8] and for phosphotransferase[1] activity.

The assay for phosphotransferase was accomplished as follows: Five grams of disodium phenylphosphate, purified[9] and 50 ml of absolute methanol were made up to 500 ml with 0.2 *N* acetate buffer at pH 5.0. 4.0 ml of this substrate solution was incubated with 1.0 ml of enzyme for 10 minutes at 37° C. The reaction was stopped with 1.0 ml of 30% trichloro-acetic acid (TCA). The protein

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precipitate was centrifuged. One ml of the supernatant liquid was used for phenol color development and 3.0 ml for phosphate color. Phenol color was developed by adding 2.0 ml of $M/2$ Na_2CO_3 and 0.5 ml of Phenol reagent[10]. The solution was brought to 5.0 ml mark with $M/5$ Na_2CO_3 and mixed. Phenol colors were read after 0.5 h development at 37° C in a Klett-Summerson colorimeter using a 660 mμ filter. Transferase activity was estimated from the difference between moles of phenol formed and moles of inorganic phosphate formed.

Uracil dissolved in the same buffer was allowed to diffuse in a similar system. Tubes were withdrawn at shorter time intervals and the agar slices were extracted with 0.1 M HCl. This compound was measured, utilizing the 260 mμ absorption in the Beckman Ultraviolet Spectrophotometer model DU.

Using another set of agar tubes (1.5% agar in 0.2 M acetate buffer at pH 5.8) and the same apparatus, a 0.1% solution of bovine hemoglobin (in pH 5.8 buffer) was allowed to diffuse. The agar was sliced and extracted with more pH 5.8 buffer. The ultra-micro method of hemoglobin analysis was employed following our own modification of the method of Mc FARLANE AND HAMILTON[11]. The hemoglobin solution is placed in a boiling water bath for 5 minutes (to destroy peroxidases). To 0.5 ml of the hemoglobin solution in a test tube, 1.0 ml of Benzidine reagent* is added, followed by 0.50 ml of 1.0% H_2O_2 (Superoxol). After mixing, the pink color is developed for 70 minutes at 15–20° C and then diluted to 10.0 ml with cold 20% acetic acid. It is read at 520 mμ in the Beckman spectrophotometer after another 8 minutes at 15–20° C. The extinction values of the colors were linear, as in the original paper for hemoglobin quantities ranging from 5–40 μg. The modification as described yielded more reproducible results and the blank values were lower than those of the original method. Also, the benzidine used did not have to be specially prepared.

Serum transferase measurements

One ml of diluted enzyme was added to 2 ml of the substrate, and the incubation mixture was buffered at pH 5.0. It contained 1% disodium phenylphosphate and 10% of either fructose, glucose, or methanol. It was incubated at 37° C for 30 minutes, and the reaction was stopped with 3.0 ml of 10% TCA.

Red cell phosphomonoesterase assay

One ml of enzyme solution was added to a 2.0 ml portion of substrate, and incubated at 30° C for 30 minutes. The substrate solution consisted of 1.5% purified disodium phenylphosphate containing 0.01 M EDTA after having been adjusted to pH 5.5 with acetic acid. The reaction was stopped with 3.0 ml 10% TCA. After filtering, 1.0 ml of filtrate was used for color development with the Phenol reagent.

Yeast phosphomonoesterase assay

This enzyme was assayed by adding a 1.0 ml aliquot from the denaturing solution to 2.0 ml of substrate. The incubation times were of 10 minutes duration at 30° C. The substrate consisted of 0.10 M sodium glycerophosphate N.F. (a mixture of α- and β-glycerophosphates. The enzyme is more active against the α-form[12]), 0.02 M $MgCl_2$ and 0.05% Triton X-100 after having been adjusted to pH 5.5 with acetic acid. The reaction was stopped with 3.0 ml 10% TCA. After filtering, 1.0 ml of filtrate was used for phosphate color development.

EXPERIMENTAL AND RESULTS

Diffusion characteristics

The set of diffusion curves obtained for the phosphomonoesterase is presented in Fig. 1. The uracil curves follow, theoretically, the type of curves obtainable for the free diffusion of dissolved particles in going from a large reservoir into a small cylinder. The theoretical method for calculating diffusion constants is to be found in the outline of GEDDES[13]. The equation used for calculating D (the diffusion constant) is given as

$$D = \frac{X^2}{4t}\left[\frac{1}{\psi^{\dagger}(1 - C/C_0')}\right]^2,$$

in which the expression $\psi^{\dagger}(1 - C/C_0')$ is the inverse function of the probability integral and can be obtained from probability integral tables[14]. C is taken as the concentration

* Our benzidine reagent is 2.0% benzidine hydrochloride (reagent grade) dissolved in 20% acetic acid containing 0.1% disodium ethylenediaminetetraacetate (EDTA).

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of diffusing substance at the level X, the distance from the agar-solution boundary. C_0' is the concentration of the diffusate in the reservoir. t is the length of time of diffusion. The concentration of the diffusate in each slice is plotted against the distance of the slice center to the boundary.

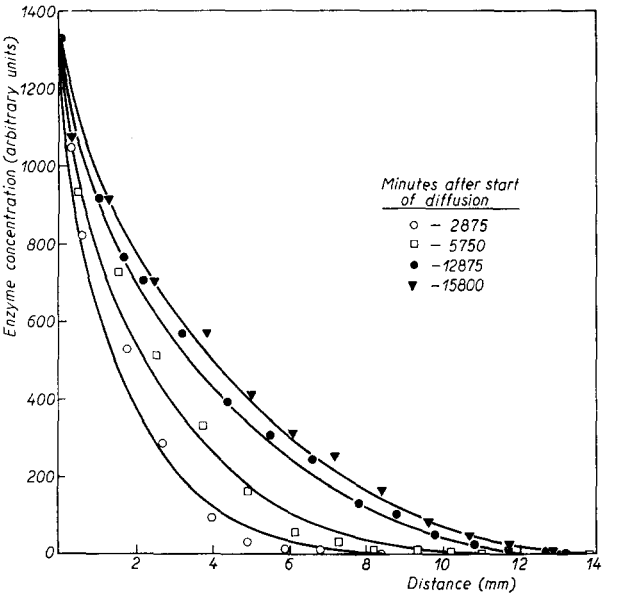

Fig. 1. Diffusion curves obtained for prostatic acid phosphatase (in aqueous medium). They demonstrate that diffusion was the means by which the enzyme entered the gel. Similar shaped curves were obtained for uracil and hemoglobin. The gel in the cylinders was equilibrated with acetate buffer (pH 5.0, 0.2 N). Two parts of this buffer, after equilibration, was mixed with one part of prostate extract before diffusion started.

The calculated D for uracil obtained from the diffusion curves approximates the D values obtained for several other compounds of nearly the same molecular weights using different methods[15,16]. For the phosphatase the calculated D, $2.2 \cdot 10^{-7}$ cm^2/sec at 1° C would indicate a molecular weight of about 300,000. This figure is an approximate average for proteins of similar diffusion constants with known molecular weights. The calculated D $1.4 \cdot 10^{-7}$ cm^2/sec for hemoglobin, somewhat lower than for the phosphatase, indicates that the phosphatase would have a lower molecular weight than the hemoglobin. The method is not suitable for the molecular weight determination of enzymes, since a more acceptable D for hemoglobin for free diffusion at 5° C is $3.94 \cdot 10^{-7}$ [17]. On the other hand, the uracil seems to diffuse freely. The data for the two proteins measured conform to ideal diffusion curves*. The uracil data indicate that diffusion occurs in agar gel without increased friction in the solvent. A network of agar in the gel may be postulated which offers sufficient hindrance to the proteins so that the effective cross section for diffusion is considerably smaller than the cross-sectional area of the tube.

In Table I are presented data for three sets of extracts of the agar tubes in the diffusion run comparing transferase with phosphatase activity for each slice. Only the data

* The criterion for ideal diffusion curves is: the best smooth lines are drawn through the points for a set time interval, as shown in the figure, and they are tested for consantt D values by selecting several arbitrary ratios of C/C_0 and measuring the corresponding D for each point.

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for higher (more reliable) activities are presented, since the transferase measurements are inherently less accurate than phosphatase measurements. The transferase data become more unreliable as the activities are lowered.

TABLE I

COMPARISON OF PHOSPHATASE AND TRANSFERASE FOLLOWING DIFFUSION

Origin to center of slice (mm)*	*Phosphatase** arbitrary unit*	*Transferase arbitrary unit*	*Ratio I*** $\frac{\text{Transferase} \times 10^3}{\text{Phosphatase}}$*	*Ratio II $\frac{\text{Transferase}}{(\text{Phosphatase} + \text{transferase})}$ (%)*
	Agar tube after 8,605 minutes of diffusion			
0.56	995	2.20	221	29.2
1.61	847	2.15	254	32.8
2.33	770	1.67	217	26.9
3.14	628	1.37	219	30.4
4.19	420	0.96	228	28.2
4.97	338	0.74	222	27.7
5.57	258	0.52	202	24.8
6.39	Sample lost		...	...
7.40	830	0.15	182	22.1
8.23	40	0.09	225	26.6
	Agar tube after 12,875 minutes of diffusion			
0.29	1050	2.40	229	25.7
0.99	922	2.02	218	26.0
1.64	770	1.56	203	25.1
2.21	710	1.44	203	28.6
3.18	565	1.34	237	29.4
4.38	388	0.87	224	25.3
5.49	306	0.76	248	27.3
	Agar tube after 15,800 minutes of diffusion			
0.34	1075	2.35	219	28.9
1.25	908	1.98	219	27.9
2.52	704	1.74	247	30.7
3.84	571	1.32	231	27.2
5.01	408	1.20	294	30.8
6.13	311	0.70	225	25.4
7.18	251	0.60	238	28.3
8.39	161	0.34	211	26.0

* Measured from weight, density and diameter of slices.

** Based on units of activity/ml corrected for mls extraction volume and mg of agar slice.

*** The justification for presenting both ratios I and II is as follows. Ratio I provides an index of how closely during diffusion, transferase follows phosphatase activity. The more constant the ratio, the greater will be the certainty of identity. Ratio II serves as a control for Ratio I. A Ratio I value is obtained from two separate samples of a single agar slice extract; one from which phosphatase activity is assayed, and the other from which transferase is assayed. On the other hand, a Ratio II value is obtained from just the transferase assay. The inorganic phosphate and phenol released at the same time are measured. In that sense variations in conditions like volumes, time, temperature and pH are eliminated. Assuming the identity of the transferase and phosphatase, large deviations from the mean of Ratio II requires placing less validity on the corresponding Ratio I.

Characteristics observed during purification

As the prostate enzyme was purified[8], each fraction was shown to contain a constant ratio of transferase to total activity using the assay procedures described.

From three patients, each having cancer of the prostate with disseminated metastases, sera containing 11, 12 and 21 Bodansky units[18] of acid phosphatase were taken

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and purified. One hundred ml aliquots of each serum were pooled and brought to 600 ml with 0.2*N*, pH 5.0 acetate buffer. The serum was then fractionated with acetone as described[8] in the procedure for purifying the prostatic phosphatase. Most of the activity was in the fraction which was soluble in 36% acetone and insoluble in 44% acetone, the same fraction which contained the prostate enzyme. Following the procedure for $Ca_3(PO_4)_2$ gel purification of prostatic phosphatase, the enzyme fraction obtained after treatment with acetone was further purified. Like the prostatic enzyme, large quantities of gel were required to adsorb the serum phosphatase, and again 0.02 *M* citrate quantitatively eluted the enzyme that was adsorbed*.

Serum assaying 950 Bodansky units of acid phosphatase was taken from a patient with disseminated cancer of the prostate. Each volume of serum was brought to 4 volumes with distilled water, adjusted to pH 5.8 with 0.2 *N* acetic acid and fractionated, as prescribed, between the limits of 36 and 44% acetone. Only 25% of the enzyme was recovered in this fraction. The precipitate volume was too large, and probably occluded and adsorbed the enzyme. When the fraction between 33.3 and 44% was collected, over 70% of the enzyme was recovered. This fraction was further fractionated, as prescribed, between the limits of 60 and 68% saturation with $(NH_4)_2SO_4$. The new fraction** contained 49% of the starting activity in the serum, and was the purified serum enzyme used in the rest of the studies.

Transferase and denaturation properties

Purified serum enzyme was diluted 25-fold and assayed for transferase activity, as described. In a similar fashion appropriately diluted enzyme, 400-fold purified from prostate glands, was tested against the three substrate systems, and Table II shows a comparison of the transferase properties of the two enzymes.

TABLE II

COMPARISON OF TRANSFERASE PROPERTIES OF ACID PHOSPHATASE FROM SERUM AND THE PROSTATE GLAND

Phosphate acceptor	*Enzyme source*	
	Serum % *transferase*	*Prostate* % *transferase*
Fructose	55	53
Glucose	37	33
Methanol	43	40

Prostatic enzyme purified 300-fold[8] was subjected to heat denaturation as in a method previously described[20]. The enzyme was diluted 10-fold for denaturation with 0.2 *N* acetate buffers at 3 different pH values, and heated at 52.4° C. The enzyme was diluted another 250-fold, after heating, with 0.2 *N*, pH 5.0 acetate buffer containing 0.02% egg albumin. The resulting solutions were then assayed for transferase and phosphatase as described. The *k* values obtained from the general equation for a first order reaction

$$2.3 \log \frac{a_f}{a_i} = -k\,(t_f - t_i),$$

* At this point there was insufficient enzyme to do further studies.

** This material was further fractionated by column chromatography using Amberlite XE-69 (Rohm and Haas). The enzyme was adsorbed from acetate buffer at pH 4.5 and was eluted at pH 6.5 using 0.05 *M* citrate. These are also characteristics for the chromatography of the enzyme from prostate (to be published) and for the enzyme from semen[19].

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are presented in Table III. Values a_f and a_i are the final and initial activities for t_f and t_i, the corresponding times of the denaturation period. The enzyme denaturation follows first-order heat denaturation kinetics[20].

TABLE III

COMPARISON OF HEAT DENATURATION CONSTANTS*

Activity	*pH 5.5*	*pH 5.0*	*pH 4.0*
Phosphatase	0.43	0.16	0.42
Transferase	0.36	0.12	0.56

* Expressed in reciprocal hours.

Samples of serum enzyme were dialyzed and diluted 5-fold with 0.2 *N* acetate buffers; one at pH 4.0, and another at pH 5.5. They were inactivated at 50.3° C. For comparison, purified prostatic acid phosphatase was treated simultaneously under similar conditions. The prostate enzyme assay was done as before. The serum enzyme was diluted another 5-fold in pH 5.0 acetate buffer after exposure to heat and then assayed. Serum from a prostatic cancer patient, assaying 74 Bodansky units of acid phosphatase, was diluted 10-fold with 0.2 *N* acetate buffer and adjusted to pH 5.5. It was subjected to heat denaturation at 52.4° C. Purified prostatic phosphatase was similarly treated. The treated serum was then further diluted 5-fold with pH 5.0 acetate buffer and assayed.

Table IV contains a comparison of the first-order heat denaturation constants of the two enzyme sources under 3 sets of conditions.

TABLE IV

COMPARISON OF HEAT DENATURATION CONSTANTS* OF THE SERUM AND PROSTATE ENZYMES

Enzyme source	*50.3°, pH 4.0*	*50.3°, pH 5.5*	*52.4°, pH 5.5*
Prostate	0.22	0.15	0.43**
Serum	0.24	0.19	0.40

* k is expressed in reciprocal hours.
** This value is taken from the corresponding denaturation shown in Table III.

In order to provide examples of how useful the heat denaturation constants can be and to obtain a relative idea of how close to identity the constants already obtained are, two other acid phosphomonoesterases were subjected to denaturation.

Human red cells were washed 5 times with 1.0% saline and then hemolyzed by adding 4 volumes of de-ionized water. The above solutions were diluted with two volumes of the appropriate 0.2 *N* acetate buffer and heat denatured in the presence of 0.01% Triton X-100 (Rohm and Haas) which protects the enzyme against surface denaturation[21]. The denaturation took place under the conditions of pH and temperature shown in Table V. One ml aliquots of denaturing enzyme solution were drawn at measured time intervals and assayed.

A crude preparation of phosphomonoesterase from autolyzed yeast obtained from Dr. K. K. TSUBOI was also subjected to heat denaturation. The filtered solutions of enzyme were 25-fold diluted with appropriate cold 0.2 *N* acetate buffers containing 0.01 *M* $MgCl_2$ (enzyme is Mg^{++} dependent[12]) and 0.006% Triton X-100. In these solutions the enzyme was denatured and 1.0 ml aliquots were taken for assay at measured time intervals.

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After establishing the linear relationships for the enzyme activities and the enzyme concentrations used, and ascertaining that first-order kinetics were followed, the k values were obtained for the red cell and yeast phosphomonoesterases.

TABLE V

COMPARISON OF HEAT DENATURATION CONSTANTS* OF THREE DIFFERENT ENZYMES**

	pH 4.0	*pH 5.0*	*pH 5.5*
Red cell	$0.77_{34.0^\circ}$	$0.48_{38.0^\circ}$	$0.22_{38.0^\circ}$
Yeast	$34.0_{30.0^\circ}$***	$1.6_{38.0^\circ}$	$0.92_{38.0^\circ}$
Prostate	$1.13 \cdot 10^{-4}{}_{34.0^\circ}$ $1.64 \cdot 10^{-5}{}_{30.0^\circ}$	$2.2 \cdot 10^{-4}{}_{38.0^\circ}$	$0.59 \cdot 10^{-3}{}_{38.0^\circ}$

* k is expressed in reciprocal hours.
** The yeast and red cell enzymes were selected only because of their ready availability.
*** This is a minimum value because the denaturation rate was too fast.

The constants obtained are compared with the prostate enzyme in Table V. The values for the latter had to be calculated from the values given for 52.4° C by the Arrhenius equation.

$$\log \frac{k_2}{k_1} = \frac{\Delta E\,(T_2 - T_1)}{2.303\,R\,T_2 T_1}.$$

The constants k_2 and k_1 are the respective first-order denaturation constants for the absolute temperatures T_2 and T_1 at which they are measured. ΔE, 89 kilocalories/mole, was obtained earlier[20]. R is the gas constant.

DISCUSSION

Differences in denaturation characteristics have been utilized to show that enzyme functions are properties of different enzymes[22, 23]. Conversely, the close fit of corresponding denaturation rate constants is perhaps the best evidence of identity of the enzymes studied. The constants of denaturation for proteins are extremely sensitive to pH and temperature[24]. In the critical regions, where the protein denaturation rate is measurable, the rate constant varies 2-fold for every 1–3° temperature change. The constant varies even more than this for single pH unit changes[20]. The variations of corresponding constants obtained for transferase and phosphatase shown in Table III could be accounted for by a 0.1° and/or 0.1 pH unit variation and are normal experimental variations.

The average deviation of constants (approximating 25%) as seen in Tables III and IV is not great considering the difficulties involved in obtaining these measurements and considering the rather large variations in k with temperature and pH. In this laboratory repetition of the procedure for obtaining these constants on the same enzyme sample allow values usually varying about 20% to be obtained. These variations are negligible when the variations among different enzymes as seen in Table V are considered. The use of heat denaturation constants with their enormous variations has hardly been exploited as a means of identifying enzymes. Compared to other physical measurement techniques it has the quality of not requiring extensive purification and the extensive range of con-

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stants as can readily be seen in Table V. The three enzymes shown could be selectively denatured and identified in the presence of one another. Problems similar to this case, multiple isodynamic enzymes, are usually the most provoking, and this treatment could be easily applied to such cases.

Diffusion and transferase data contribute to showing the identity of the serum and prostate enzyme. By surveying the literature[25,26], lists have been prepared for diffusion data of many proteins. Very few protein groups exist with the same diffusion constant. Constants identical to two significant figures for two enzymes would be required to explain Ratio II of Table I for simple diffusion if the two enzymes were not the same. This requires either two molecules of the same molecular weight and symmetry or molecular weights and symmetries dissimilar but capable of diffusing together. Since the evidence presented suggests an agar network, the method used would tend to eliminate the latter possibility. The network would provide more discrimination than free diffusion with regard to molecular volume and asymmetry. If a more open gel structure could be made the method could be suitable for molecular weight determinations below 100,000.

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SUMMARY

The identity of a hydrolase and a transferase activity was shown. Identity of diffusion characteristics helped to establish prostatic phosphotransferase and acid phosphomonoesterase as the same enzyme. By utilizing purification characteristics, common transferase activities, and denaturation kinetics, the identity of the serum acid phosphatase enzyme in advanced cancer of the prostate and the normal prostate enzyme has been established. Differences that were very great were shown for the denaturation kinetics for enzymes which normally are functionally similar. Characteristics of agar diffusion of small and large molecules have been presented, and a model technique for the use of this process in establishing enzyme identities was described.

RÉSUMÉ

Les auteurs ont démontré l'identité entre une activité hydrolasique et une activité transférasique. L'identité des caractéristiques de diffusion a servi à établir que la phosphotransférase prostatique est le même enzyme que la phosphomonoestérase acide. A l'aide des caractéristiques de purification, des activités transférasiques communes et des cinétiques de dénaturation, l'identité de la phosphatase acide du sérum dans un cancer avancé de la prostate et de l'enzyme de la prostate normale a été démontrée. De très grandes différences dans les cinétiques de dénaturation ont été observées chez des enzymes qui, normalement, sont fonctionnellement semblables. Les caractéristiques de diffusion dans l'agar de petites et de grosses molécules sont présentées et une technique standard d'emploi de cette méthode pour établir l'identité d'enzymes est décrite.

ZUSAMMENFASSUNG

Die Identität einer Hydrolasen- und einer Transferasenaktivität wurde bewiesen. Die Identität der Diffusionseigenschaften konnte bei der Feststellung benutzt werden, dass Phosphotransferase und saure Phosphomonoesterase aus der Prostata ein und dasselbe Enzym darstellen. Auf Grund der Purifikationseigenschaften, der gewöhnlichen Transferaseaktivitäten und der Denaturierungskinetik, wurde die Identität des sauren Phosphatase-Enzyms aus dem Serum bei vorgeschrittenem Krebs der Prostata mit dem normalen Enzym der Prostata festgestellt. Es wurde festgestellt: dass

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sehr grosse Unterschiede in der Denaturierungskinetik von Enzymen bestehen, welche normalerweise funktionelle Aehnlichkeit aufweisen. Die Eigenschaften der Agardiffusion von kleinen und grossen Molekülen wurden untersucht und eine Modelltechnik für die Anwendung dieses Prozesses auf die Identifizierung von Enzymen beschrieben.

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