View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by The Research Repository @ WVU (West Virginia University)

DEVELOPMENT OF A RAPID ASSAY FOR PROLYL HYDROXYLASE IN MOUSE LUNG

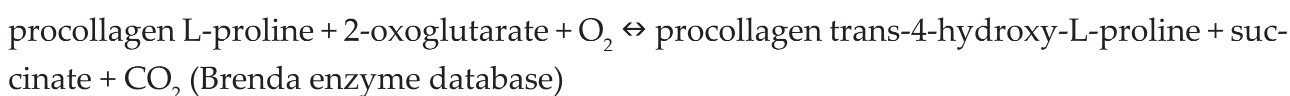
Nathan Pearson, with Dr. Dale Porter
and Dr. Kenneth P. Blemmings

Abstract

The National Institute for Occupational Safety and Health is conducting research on the effects of respiratory exposure to carbon nanotubes. Exposure to some kinds of carbon nanotubes has been associated with a fibrogenic response in lungs that has negative effects on lung physiology and human health. A thorough understanding of the molecular events leading to fibrosis could offer prophylactic or therapeutic approaches to avoid the fibrosis. Several different enzymes are associated with fiber formation in the lung ,and one of interest is prolyl hydroxylase (PH-4). Current protocols for measuring PH-4 activity are expensive, cumbersome, and time-consuming. A rapid assay protocol would aid in our understanding of the regulation on the enzyme's activity. To prepare the tissue for assay, it was homogenized and then microsomes were prepared by differential centrifugation. Then, a surfactant was used to solubilize the protein, allowing substrate access. The incubation occurred in stoppered vials. The vials were placed in a 37 degree C water bath with shaking. A radioactive co-substrate for the reaction, 2-oxo[1-^{14}C]-glutarate, was incubated in the presence and absence of a synthetic peptide containing proline and the liberated $^{14}CO_2$ was captured. The reaction was terminated by adding pH 5 phosphate buffer to the reaction vial. Radioactivity was determined using liquid scintillation spectrometry. The peptide-dependent $^{14}CO_2$ captured was used to estimate enzyme activity. This assay has been determined to be linear with respect to enzyme concentration as well as incubation time. This is a useful method as it can be completed in a matter of hours and requires no previous preparation of tissue or substrate. This rapid assay will be used to assess PH-4 in mouse lung from mice exposed or not exposed to carbon nanotubes. PH-4 regulation, or a lack thereof, after nanotube exposure suggests the molecular pathway by which the fibrogenic response associated with carbon nanotube exposure is elicited.

Introduction

Carbon nanotubes are cylindrical carbon molecules with diameters on the order of nanometers and lengths on the order of micrometers (Chen et al. 2000). Carbon nanotubes are important because they have several novel and potentially useful properties making them desirable for many industrial and commercial applications. The manufacture of carbon nanotubes is increasing, and as such they are coming under increased toxicological scrutiny (Donaldson et al. 2006). Because of their size and physical properties, it is suspected that the toxicity of carbon nanotubes may be similar to that of asbestos (Prosie et al. 2008). Part of the physiological response elicited by asbestos fiber exposure is collagen deposition in the lung tissue (Wrzaszczyk and Owczarek 1996). PH-4 is an enzyme necessary for the production of collagen. It is a 2-oxoglutarate dioxygenase and catalyzes the reaction:


procollagen L-proline + 2-oxoglutarate + O_2 ⇔ procollagen trans-4-hydroxy-L-proline + succinate + CO_2 (Brenda enzyme database)

The enzyme decarboxylates 2-oxoglutarate. Also, one oxygen atom from the O_2 becomes incorporated into the succinate while the other oxygen atom from the O_2 becomes incorporated into the proline to generate 4-hydroxyproline. The enzyme is necessary for collagen production because the hydroxyl groups on the 4-hydroxyprolyl residues stabilize the triple helix of collagen under physiological conditions. The enzyme cannot interact with free proline or with proline occurring in just any amino acid sequence. Rather, it requires a minimum X-Pro-Gly triplet to interact with the proline. The intracellular location of PH-4 is within the cisternae of the rough endoplasmic reticulum. The enzyme requires Fe^{2+} and ascorbate, and maximal enzyme activity requires dithiothreitol, bovine serum albumin, and catalase (Kivirikko and Myllylo 1982).

The National Institute for Occupational Safety and Health (NIOSH) is currently conducting research on the effects of respiratory exposure to carbon nanotubes. Early results have demonstrated that some kinds of carbon nanotubes do elicit a fibrogenic response that leads to health problems (Lam et al. 2006; Shvedova et al. 2005). NIOSH is interested in determining the molecular pathway by which this fibrogenic response occurs. A change in the regulation of PH-4, or a lack thereof, after nanotube exposure suggests the molecular pathway by which the fibrogenic response occurs. For example, if PH-4 is upregulated it may indicate collagen deposition and thus a fibrogenic pathway similar to that of asbestos. Our role in NIOSH's larger study will be to determine if PH-4 regulation is altered in mouse lung after carbon nanotube exposure.

Several assays have been developed to measure the activity of PH-4. The assays use either radiolabeled biologically prepared substrate or a synthetic polypeptide substrate (as the source of procollagen-L-proline). The assays involving radiolabeled biologically prepared substrates

are the most sensitive, and in most reported cases only these methods can be used to accurately determine enzyme activity from crude tissue extracts (Kivirikko and Myllylo 1982). Unfortunately, these assays are expensive, cumbersome, and time-consuming. The assays using synthetic polypeptide substrates are less expensive and faster, but they have lower sensitivity and in most cases can only be used with partially purified enzyme.

For this particular project a quick, inexpensive assay that could be used with crude tissue extract was desired. By adapting previously published results (Kao et al. 1975; Kivirikko and Myllylo 1982), such an assay has been developed, and it will allow us to determine whether PH-4 regulation is altered after carbon nanotube exposure.

Methods and Calculations

Assay Overview

The components necessary to carry out the reaction (enzyme extract, peptide substrate, labeled 2-oxoglutarate, and cofactors) were added to a 3 mL reaction vial. The labeled 2-oxoglutarate was added last to begin the reaction. The reaction vial was then incubated at 37 degree C for 20 minutes. The reaction was ended by injecting pH 5 phosphate buffer into the reaction vial. The reaction vial was then shaken for 30 minutes to release the evolved CO_2. A base trap suspended in the reaction vial collected the evolved CO_2. This base trap was then placed in a liquid scintillation vial and counted.

Preparation of Assay Components

Crude enzyme extract was obtained from mouse lung. After the mice were sacrificed, their lungs were immediately harvested, weighed, and homogenized in enzyme buffer (Kao et al. 1975) (.2 M NaCl, .1 M glycine, 50μM DTT, .01M Tris-HCl, pH 7.8) using a Potter-Elvehjem tissue grinder and approximately 1mL buffer per gram of tissue. Depending on the particular experiment being done, between 1 and 5 mice were used. When multiple mice were used, their homogenized lungs were combined into one homogenate. The homogenate was then centrifuged in 1.5mL Eppendorf tubes at 10,000 x g for 20 minutes. The supernatant was then collected and put on ice. The pellet was resuspended in .5mL NaCl buffer and again centrifuged at 10,000 x g for 20 minutes. This supernatant was collected and added to the previously collected supernatant. The combined supernatant was then centrifuged at 37,000 x g for 30 minutes. The resulting pellet

was then collected (representing the microsomal fraction) and resuspended in the NaCl buffer. Triton X-100 (0.1 %) was added and the solution was placed on ice.

While centrifuging the enzyme extract, a "Master Mix" of enzyme cofactors and activators was prepared and kept on ice. This "Master Mix" consisted of 20mM ascorbic acid, 1mM $FeSO_4$, 1mM DTT, 20mg/mL bovine serum albumin, and 2 mg/mL catalase (Kivirikko and Myllylo 1982). The amount of Master Mix prepared was determined by the number of trials to be run (.333mL/trial).

The peptide substrate (Pro-Pro-Gly)10 • 9 H_2O was diluted to 1 mg/mL in distilled water and then heated at 100 degrees C for 10 minutes. After heating, the peptide was placed on ice. The amount of peptide prepared was determined by the number of trials to be run (.1mL/trial).

A solution of 2mM 2-oxoglutarate was prepared. The amount of 2-oxoglutarate prepared was determined by the number of trials to be run (.05mL/trial). Additionally, 20µL of 2-oxo-[1-^{14}C]glutarate (56.8mCi/mmol) was added to the 2-oxoglutarate solution. This solution was then placed on ice.

Trial Preparation

The reaction was carried out in 3 mL vials. These vials were kept on ice as the assay components were added. A 5mm boiling bead was placed in each reaction vial. Master Mix (.333mL) and enzyme extract (.3mL) were added to each reaction vial.

A base trap was prepared to capture the evolved CO_2. The base trap consisted of methyl cellusolve and ethanolamine in a 2:1 ratio. Base trap (.45mL) was added to a .5mL Eppendorf tube that was then suspended inside of the reaction vial.

Figure 1

Peptide solution (.1mL) was added to each treatment vial and (.1mL) distilled H_2O was added to each control vial.

In Figure 1, the 3mL reaction vial with .5mL Eppendorf tube suspended from the stopper. The Master Mix, 2-oxoglutarate, and peptide substrate are injected into the bottom of the reaction vial. The base trap is placed in the Eppendorf tube and collects the evolved CO_2.

Assay

Labeled 2-oxoglutarate (.05mL) was then added to each vial to start the reaction. Each vial was then immediately stoppered with the base trap suspended from a wire attached to the stopper. The vials were then incubated at 37 degrees C with shaking for 20 minutes. The reaction was ended in each vial by adding .5mL of pH 5 phosphate buffer (1 M KH_2PO_4) via a syringe inserted through the stopper.

The vials were then taken out of the water bath and shaken for 30 minutes at room temperature.

The Eppendorf tube containing the base trap was then added to a counting vial along with a scintillation cocktail and counted.

Calculation of Enzyme Activity

From the scintillation spectrometer we get a DPM (disintegrations per minute) value representing the labeled CO_2 collected in the base trap. This DPM value is converted to a CO_2 (and thus an enzyme activity) level by comparing it to the DPM from a labeled 2-oxoglutarate solution of known concentration. For enzyme specific activity, the DPM difference between the treatment trials and the control trials is used.

The calculation for enzyme specific activity is then:

(Treatment DPM – Control DPM) * ((moles 2-oxoglutarate)/(2-oxoglutarate vial counts)) = moles labeled CO_2

This result is divided by the amount of mouse tissue in each sample to get a unit for enzyme activity that has units of moles CO_2 evolved/g mouse tissue.

To optimize the assay in various respects the experiments shown below in table 1, table 2, and table 3 were performed. These experiments optimized the Master Mix volume, the size of the reaction vessel, and the rate of shaking during incubation. In each of these experiments, the procedure used was very close to the one just described.

Results

For all experimental results, the raw data came in the form of Disintegrations Per Minute (DPM). Enzyme specific DPM was obtained by taking the difference between trials run with the enzyme's peptide substrate ((Pro-Pro-Gly)10 • 9 H_2O) and trials run without it. This enzyme specific DPM can then be converted to enzyme activity with units of moles CO_2 evolved per gram tissue.

Table 4 and Figure 2 demonstrate the linearity of our assay with respect to differing enzyme concentration. Table 5 and Figure 3 demonstrate the linearity of our assay with respect to time.

Table 1 :Effect of Variations in Master Mix Volume

Master Mix Volume	.1666 mL peptide	.1666 mL no peptide	.333 mL peptide	.333 mL no peptide	.5 mL peptide	.5 mL no peptide
	1154.23	1011.11	1358.28	564.5	654.09	987.65
	1023.34	645.6	1221.34	1051.12	1768.8	795.26
	678.87	659.12	1477.24	908.43	1245.56	1654.23
	1409.23	605.72	1332.98	1013.13	1521.23	1256.67
	899.45	1432.3	1003.32	1066	1165.34	1340.48
	1890.21	1222.9	1060.43	998.08	432.23	543.7
	1722.39	983.34	1232.21	955.65	976.45	923.21
	1300.23	1450.08	1278.33	843.34	1343.75	459.47
Average	1259.74375	1001.27125	1245.51625	925.03125	1138.43125	995.08375
Enzyme specific DPM	258.4725		320.485		143.3475	
Standard Deviation	408.789206	345.7461947	155.2949729	163.3302627	440.4219828	406.7255518
T-Test	0.194224355		0.00126636		0.509929937	

This experiment was done using the 3mL reaction vial and shaking 110r/min. The Master Mix volume used in Kao et al. (1975) is .5mL. In this project smaller amounts of tissue are used, so it was decided to concentrate the Master Mix in an attempt to lower the reaction volume and obtain more precise results. The above data shows that lowering the Master Mix volume to .333mL increases the precision of the assay over the precision achieved when using Master Mix volume .5mL or .1666mL.

Table 2: Effect of Changing The Size of The Reaction Vessel

#(New) 3 mL reaction vial peptide	(New) 3 mL reaction vial no peptide	(Original) 25 mL Erlenmeyer peptide	(Original) 25 mL Erlenmeyer no peptide
539.6	343.6	358.4	187.4
625.6	383.5	542.8	333.2
635.7	349.1	486.4	535
541.7	358.1	677.9	301.2
411.4	443.6	827.5	491.9
604.9	445.1	482.3	565.1
401.7	348.4	664.4	558.2
622.5	377.7	861.4	866.1
446.6	331.7	709.3	543.1
584.2	354.1	456.67	
average	average	average	average
541.39	373.49	606.707	486.8
standard deviation			
90.57218668	40.32968165	166.9140732	196.3087364
Enzyme Specific DPM		Enzyme Specific DPM	
167.9		119.907	
ttest new		ttest old	
0.000152392		0.173208502	
Coefficient of variance	Coefficient of variance	Coefficient of variance	Coefficient of variance
16.72956403	10.79806197	27.5114797	40.32636328

This experiment was done using .5mL Master Mix and shaking 110r/min. Once again, due to the small amounts of tissue being used in this project, it was decided to lower the volume of the reaction vessel to try to cause more efficient mixing of the assay components and thus increase the precision of the assay. The above data shows that lowering the volume of the reaction vessel (from 25mL to 3mL) does increase the precision of the assay.

Table 3: Effect of Changing the Rate of Shaking During Incubation

shaking rate (r/ min)	50 peptide	50 no peptide	80 peptide	80 no peptide	110 peptide	110 no peptide
	1065.3	934.21	1078.12	956.65	1240.1	954.23
	1034.9	854.06	855.5	1190.22	1168.89	904.45
	876.54	1260.75	980.1	860.98	1300.4	654.15
	665.2	565.4	1184.46	544.4	988.89	1023.2
	1209.92	875.2	1353.3	990.02	1002.24	838.26
	1250	920.43	1109.23	1105.5	1037.72	906.5
Average	1016.976667	901.675	1093.451667	941.295	1123.04	880.1316667
Enzyme Specific DPM	115.3016667		152.1566667		242.9083333	
Standard Deviation	218.0149879	221.9883426	170.7379775	226.0588329	132.0077275	126.5438513
T-test	0.385384722		0.219793911		0.008684604	

This experiment was done using the 25mL Erlenmeyer flask and .5mL Master Mix. Increasing the rate of shaking during incubation more thoroughly mixes the assay components and increases the precision of the assay. Also, the enzyme specific DPM appears to increase with faster shaking. This is an added benefit, as higher enzyme specific DPM will make it easier to identify differences in enzyme specific DPM between individual mice.

Table 4: DPM With and Without Peptide Substrate and at Differing Enzyme Concentration

	[1] peptide	[1] no peptide	[.5] peptide	[.5] no peptide	[.25] peptide	[.25] no peptide
	1518.03	871.9	733.1	431.4	405.8	374.7
	2403.81	776.8	607.6	524.9	332.4	350.3
	1355.81	781.3	641	376.9	410.8	376.6
	1211.08	930	247.1	265.9	500.3	306.2
	1535.73	839.3	878.5	402.7	385	358.3
	952.4	1048.69	551.1	184.8	374.1	
average	1496.143333	874.665	609.7333333	364.4333333	401.4	353.22
Enzyme Specific DPM	621.4783333		245.3		48.18	
t-test	0.026679563		0.039117194		0.104464231	
standard deviation	494.4727028	102.8587116	211.3659449	121.516084	55.97174287	28.51853783

In this experiment, the assay was performed at standard enzyme concentration (represented by [1]) and also at one-half and one-fourth normal enzyme concentration ([.5] and [.25]). The t-tests compare the trials with the peptide substrate added for a given enzyme concentration to the trials without the peptide substrate added for that enzyme concentration.

In Figure 2: Graphical representation of the data from Table 4. Here the difference in DPM between Treatments (peptide) and Controls (no peptide) has been converted to enzyme activity with units of nanomoles CO2 evolved per minute.

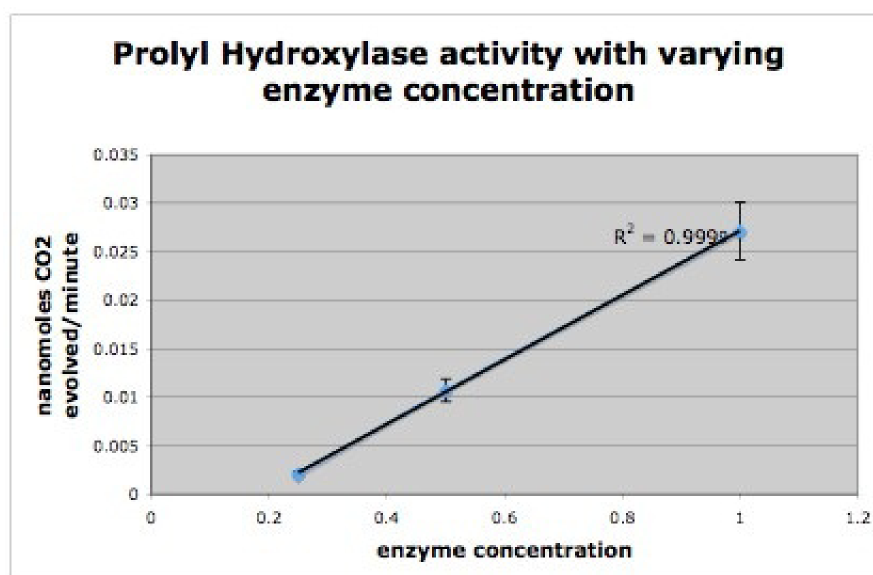


Figure 2

Table 5: DPM With and Without Peptide Substrate and with Respect to Time

	5 minutes peptide	5 minutes no peptide	20 minutes peptide	20 minutes no peptide	60 minutes peptide	60 minutes no peptide
	706.2	348.4	2087.89	1512.58	2780.51	1557.67
	668.3	289.6	2190.91	1775.27	3155	1985.94
	597.4	193.1	2066.19	1422.98	3681.09	1886.92
	670.1	406.65	1789.29	1418.44	2580.77	2325.58
	747.8	355.56	1983.23	1467.04	2637.9	2229.12
Average	677.96	318.662	2023.502	1519.262	2967.054	1997.046
T-test	0.003374733		0.13618652		0.005486779	
Enzyme specific DPM	359.298		504.24		970.008	
Standard Deviation	55.49804501	81.5446425	150.3916664	148.1107539	457.5826309	302.9419413

In this experiment the reaction was allowed to run for 5, 20, or 60 minutes.

Figure 3 is a Graphical representation of the data from Table 5. Here the difference in DPM between Treatments (peptide) and Controls (no peptide) has been converted to enzyme activity with units of nanomoles CO_2 evolved per gram of lung.

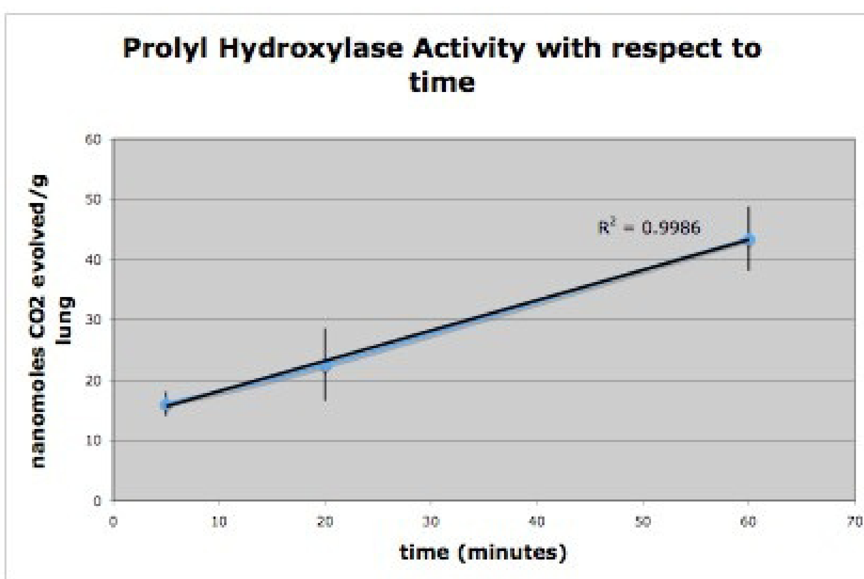


Figure 3

Discussion

The assay developed here is based on previously published results (Kao et al. 1975; Kivirikko and Myllylo 1982). In previous studies, researchers were concerned with demonstrating that a PH-4 assay that measures enzyme activity by following the release of $^{14}CO_2$ is reliable. In developing a PH-4 assay for use in the NIOSH project we

had the added requirement that the assay be able to be used to determine whether or not a statistical difference exists between the PH-4 activities of the lungs of individual mice. This meant that the assay needed to be able to give reliable enzyme activities using small amounts of tissue (a single mouse lung weighs ~.1g). In previously reported results working with small amounts of tissue was not a concern. For example, in Kao et al. (1975) 5g of tissue would typically be used per assay (the 5g value came from combining tissue from many subjects).

Having to work with a small amount of tissue means a couple things. Obviously, less tissue means less enzyme and thus less activity for us to measure. At low levels of activity it becomes difficult to obtain a statistically significant result. Further, because there is some inherent variability in the DPM for any given trial using this assay (note the large standard deviations in the Results section) it is necessary to run several treatment and control trials to get a sufficiently precise measurement of enzyme activity. Thus, our problem was that we needed to find a way to run several trials that produced DPM numbers high enough to be of use using a small amount of tissue.

The first step taken in this direction was to add an additional round of centrifugation at 10,000 x g in order to retain more of the microsomal fraction (PH-4's subcellular location is the endoplasmic reticulum). By next centrifuging the resulting supernatant at 37,000 x g and taking the pellet, the microsomal and mitochondrial fractions were separated. This is a useful step as 2-oxoglutarate is also a substrate for the oxoglutarate dehydrogenase complex of the citric acid cycle which occurs in the mitochondria. The result of this separation is that a larger percentage of the total activity during the incubation period is enzyme specific.

The surfactant Triton X-100 is used to solubilize PH-4 and allow it access to its peptide substrate. Rather than include the surfactant in the homogenization buffer as is done in the literature, we elected to add the surfactant after the microsomal fraction is collected. Adding the surfactant at this time reduces the chance that unwanted enzymes that will interfere with data collection will also be solubilized.

These changes in the centrifuging and solubilization procedure were necessary to obtain enzyme specific activity. That is, the difference between treatment and control trials was statistically insignificant before making these changes to the assay.

Again due to the small amount of tissue being used, we elected to further alter the assay as described in the literature by using a smaller amount of Master Mix solution, carrying out the reaction in a smaller vessel, and shaking the reaction vials more vigorously during incubation. The benefit of these changes is shown in Tables 1, 2, and 3, respectively. Using .333mL as the volume for the Master Mix resulted in the highest enzyme specific DPM and the lowest standard

deviation. The .333mL Master Mix also returned the lowest T-test result. Using the 3mL reaction vial instead of the 25mL Erlenmeyer flask to carry out the reaction lowered the standard deviation and returned a lower T-test value. Finally, shaking the reaction vial more vigorously during incubation (110r/min as opposed to 80 or 50) lowered the standard deviation and returned a lower T-test value.

This assay has been demonstrated to be linear with respect to both enzyme concentration (Table 4, Fig. 2) and time (Table 5, Fig. 3). These results are important as they demonstrate that the assay gives results that fit basic biochemical theory.

The assay is quick, easy, and inexpensive. It requires no previous preparation of tissue or substrate and can be completed from start to finish in a little over 5 hours. Reliable results have also been obtained breaking up the assay into two parts. After the last centrifugation step the enzyme extract can be placed on ice overnight and used the next day. This makes the assay even more convenient, as it creates two approximately three hour work sessions.

After much troubleshooting and tweaking, the assay is now at a point where it can be used for its intended purpose, determining whether or not carbon nanotube exposure alters PH-4 regulation in mouse lung.

WORKS CITED

Brenda enzyme database 2008. 18 Oct. 2008 <http://www.brenda-enzymes.info/php/result_flat.php4?ecno=1.14.11.2>

Chen, P., J. Lin, and K.L. Tan. 2000. Carbon Nanotubes: A Future Material of Life. *International Union of Biochemistry and Molecular Biology Life* 49(2): 105-8

Donaldson, K., R. Aitken, L. Tran, V. Stone, R. Duffin, G. Forrest, and A. Alexander. 2006. Carbon nanotubes: a review of their properties in relation to pulmonary toxicology and workplace safety. *Toxicological Sciences* 92(1): 5-22.

Kao, W., R. Berg, and D. Prockop. 1975. Ascorbate increases the synthesis of procollagen hydroxyproline by cultured fibroblasts from chick embryo tendons without activation of prolyl hydroxylase. *Biochimia et Biophysica Acta* 411: 202-215

Kivirikko, K.I., and R. Myllylo. 1982. Posttranslational enzymes in the biosynthesis of collagen: intracellular enzymes. *Methods in Enzymology* 82: 245-304.

Lam, C.W., J.T. James, R. McCluskey, S. Arepalli, and R.L. Hunter. 2006. A review of carbon nanotube toxicity and assessment of potential occupational and environmental health risks. *Critical Review of Toxicology* 36(3):189-217.

Prosie, F., F.X. Lesage, and F. Deschamps. 2008. Nanoparticles: structures, utilizations and health impacts. *Presse Med* 37(10): 1431-7.

Shvedova, A., E. Kisin, R. Mercer, A. R. Murray, V. Johnson, A. Potapovich, Y. Tyurina, O. Gorelik, S. Arepalli, D. Schwegler-Berry, A. Hubbs, J. Antonini, D. Evans, B. Ku, D. Ramsey, A. Maynard, V. Kagan, V. Castranova and P. Baron. 2005. Unusual inflammatory and fibrogenic pulmonary responses to single-walled carbon nanotubes in mice. *American Journal of Physiology: Lung Cellular and Molecular Physiology* 289(5): 698-708.

Wrzaszczyk, B., and H. Owczarek. 1996. Relationship between the physiochemical properties of asbestos and pulmonary fibrosis. *Med Pr*. 47(4): 401-9.