EXPERIMENTAL

CHAPTER 3

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3.1. MATERIALS:

3.1.1. Chemicals: The following chemicals and biochemicals were used during the present investigation:

3.1.1.1. *For gel electrophoresis:* Acrylamide, N,N-methylene-bis-acrylamide, N,N,N,N-tetramethylethylenediamine, sodium dodecyl sulphate (SDS) and silver nitrate were from Sisco Research Laboratories, Mumbai; ammonium persulphate and sodium thiosulphate were from E. Merck, India; glycine and 2-mercaptoethanol were from Loba Chemie, Mumbai; acetic acid and

methanol from Qualigens Fine Chemicals (a division of Glaxo India Limited), Mumbai; tris (hydroxymethyl) aminomethane (Tris) was obtained from HiMedia Laboratories Pvt. Ltd., Mumbai.

3.1.1.2. *For carbonic anhydrase assay:* Bovine erythrocyte carbonic anhydrase (EC 4.2.1.1) [lyophilized powder] (Product No. C3934], 4-Nitrophenyl acetate (Product No. N8130) and p-nitrophenol were purchased from Sigma Chemical Co., USA; Disodium hydrogen orthophosphate anhydrous and acetone were from Qualigens Fine Chemicals (a division of Glaxo India Limited), Mumbai; Hydrochloric acid from Glaxo India Ltd., Mumbai; and sodium chloride from Central Drug House, New Delhi.

3.1.1.3. *For protein estimation:* Copper sulphate was from BDH, Mumbai; sodium carbonate was from Qualigens Fine Chemicals (a division of Glaxo India Limited), Mumbai; sodium hydroxide from HiMedia Laboratories Limited, Mumbai; Rochelle salt (sodium potassium tartarate) was obtained from Loba Chemie, Mumbai; and Folin-Ciocalteau reagent was obtained from Sisco Research Laboratories, Mumbai.

3.1.1.4. *For preparation of apocarbonic anhydrase:* Bovine erythrocyte carbonic anhydrase (EC 4.2.1.1) [lyophilized powder] (Product No. C3934] was purchased from Sigma Chemical Co., USA;

Pyridine 2, 6-dicarboxylic acid (MW 167.12) (Product No. 800614) was from Merck, Germany.

3.1.1.5. *For re-activation of apocarbonic anhydrase:* Commercial zinc atomic absorption standard solution [$Zn(NO_3)_2$ in HNO_3] (Catalogue No. 119806) was purchased from Merck, Germany.

3.1.1.6. *Other chemicals:* All other chemicals and biochemicals were of analytical reagent grade.

3.1.1.7. *Water:* Purified deionized water used in the investigation was prepared using the water purification system TKA Smart2Pure UV/UF (Germany).

3.1.2. Solutions, buffers and reagents: The following solutions, buffers and reagents were used in the present investigation:

3.1.2.1. *Enzyme (bovine erythrocyte carbonic anhydrase) solution:* 10 mg of the lyophilized powder of bovine erythrocyte carbonic anhydrase (EC 4.2.1.1) was dissolved in 2mL of the suspension buffer (0.2M phosphate buffer pH 7.4).

3.1.2.2. *Substrate solution (6.0 mM p-nitrophenyl acetate):* 13.8mg p-nitrophenyl acetate was dissolved in 1.5mL of acetone and volume made up to 12.5mL by slowly mixing with deionized water.

3.1.2.3. *Suspension buffer:* 0.2M phosphate buffer pH 7.4

3.1.2.4. *Assay buffer:* 0.5M phosphate buffer pH 7.5

3.1.2.5. *Zinc standard solution:* Working standards were prepared from the commercial zinc atomic absorption standard stock solution mentioned in *For re-activation of apocarbonic anhydrase* by diluting with appropriate buffer.

3.1.2.6. *Trichloroacetic acid solution:* 5% (v/v) Trichloroacetic acid (TCA)

3.1.3. Main instruments used in the investigation: The two main instruments used in the investigation were:

(i) Atomic absorption spectrophotometer (AAS): The determinations of zinc in serum and other samples were performed with Atomic Absorption Spectrophotometer (AAS) Perkin Elmer 3110 shown in **Fig.1** installed in Sophisticated Analytical Instrument Facility, NEHU, Shillong (Estimation parameters: Wavelength, 213.9nm; Slit width, 0.7nm; Gases used, air-acetylene; Sensitivity, 0.011ppm; Detection limit, 0.0008ppm).

(ii) UV-Visible spectrophotometer: Enzymatic zinc determination and other colorimetric determinations were performed with UV-Visible Spectrophotometer Hitachi U-2001.

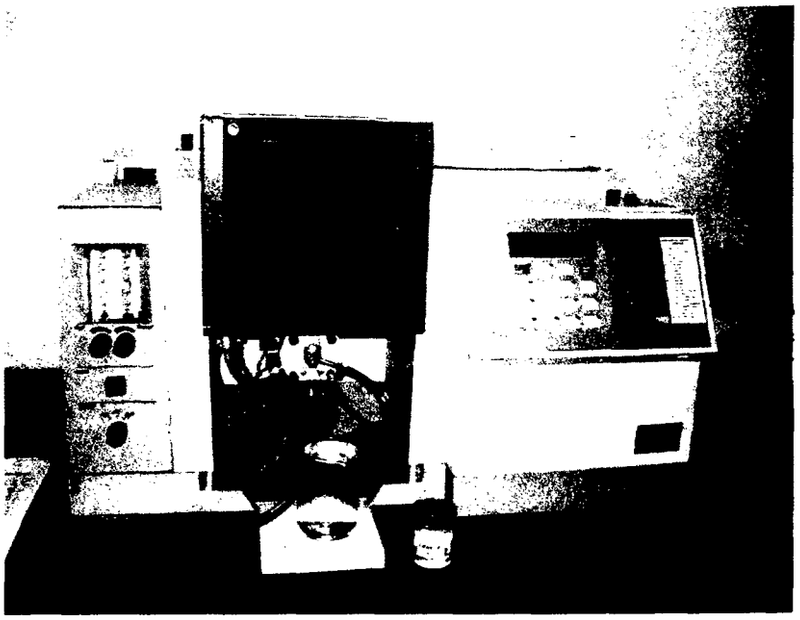

Fig.1: Atomic absorption spectrophotometer Perkin Elmer 3110 used for serum zinc determination installed in Sophisticated Analytical Instrument Facility, NEHU, Shillong (Estimation parameters: Wavelength, 213.9nm; Slit width, 0.7nm; Gases used, air-acetylene; Sensitivity, 0.011ppm; Detection limit, 0.0008ppm)

3.2. METHODS

3.2.1. ***Testing the homogeneity of the commercial bovine erythrocyte carbonic anhydrase preparation:*** As homogeneity is an essential feature for application of an enzyme in kinetic studies, the purity of the commercial bovine erythrocyte carbonic anhydrase preparation was tested by polyacrylamide gel electrophoresis in presence of sodium dodecyl sulphate (SDS-PAGE) coupled with silver staining of the separated protein species:

3.2.1.1. *Polyacrylamide gel electrophoresis in presence of sodium dodecyl sulphate:* The purified lectin preparation was subjected to polyacrylamide gel electrophoresis in presence of sodium dodecyl sulphate (SDS-PAGE) according to the procedure of Laemmli (1970). The gel electrophoresis system consisted of two contiguous but distinct gels - a resolving (lower) gel and a stacking (upper) gel. The gel casting apparatus was assembled after thoroughly cleaning the glass plates, spacers, comb and buffer reservoirs. The lower end of the gel space was plugged with 1% agar solution. The polymerizing mixture for resolving gel (12%) was prepared by mixing together 3.2mL of 30% acrylamide solution (29.2g acrylamide and 0.8g N,N-methylene-bis-acrylamide dissolved in 100mL of distilled water), 3.0mL of resolving gel buffer (1.0M Tris-HCl pH 8.8), 0.08mL of 10% SDS solution and 1.71mL of distilled water. After degassing with a vacuum pump, the polymerizing mixture was thoroughly mixed with 37.5µL of 100mg/mL of ammonium persulphate solution and 6.2µL of

N,N,N,N-tetramethylethylenediamine (TEMED) and then quickly put between the glass plates up to the mark delimiting the resolving gel. After putting a layer of water on the top, the gel was left to set for 30-60min. Meanwhile monomer solution of the stacking gel (5%) was prepared by mixing together 1.0mL of 30% acrylamide solution, 0.75mL of stacking gel buffer (1.0M Tris-HCl pH 6.8), 0.06mL of 10% SDS solution and 4.19mL distilled water. After deaeration, the monomer solution was mixed with appropriate amount of ammonium persulphate (75.0µL of 100mg/mL solution) and TEMED (6.0µL). After pouring off the water over the resolving gel, top of the gel was rinsed with a little volume of stacking gel solution. Then, the stacking gel solution was put into the space above the resolving gel, well-formed comb was placed in the stacking gel, and the gel was left to set for 30-60min. After the polymerization of the stacking gel, the buffer reservoirs were filled with electrode buffer (prepared by dissolving 3.0g of Tris, 14.4g glycine and 1.0g SDS in a total volume of 1L of distilled water) and the comb was removed carefully without distorting the shape of the wells. The sample solution was prepared by mixing together two volumes of protein solution containing appropriate amounts, one volume of 20% 2-mercaptoethanol and one volume of sample buffer (x4) prepared by mixing 2.5mL of 1.0M Tris-HCl buffer pH 6.8, 3.2mL of 25% SDS, 4.0mL of 100% glycerol and 0.3mL of 33.3mg/mL bromophenol blue solution. The sample solution was taken in a small test tube and heated in a boiling water bath for

5min, cooled to room temperature, and then loaded on to the well of stacking gel using a microlitre Hamilton syringe. Finally the electrophoresis unit was connected to a power supply in such a way that the lower electrode was made anode and the upper one, the cathode. The current was adjusted to 15mA until the dye front traveled through the stacking gel. Then, the current was increased to 25mA and the electrophoresis was continued until the dye front reached the bottom of the resolving gel. After the run was complete, the power supply was disconnected. The gel was carefully removed from the glass plates and then stained by the procedure described below.

3.2.1.2. *Silver staining of protein on SDS-PAGE:* The proteins separated on polycrylamide gel electrophoresis in presence of sodium dodecyl sulphate were stained by a modification of the method of Merril (1990). The successive steps involved in the staining procedure are given below:

(i) The SDS-PAGE gel was fixed by soaking overnight in a solution containing 50% methanol, 12% acetic acid (glacial) and 0.075% formaldehyde.

(ii) The gel was washed by soaking in 50% ethanol with occasional shaking, and then subsequent decantation of the ethanol solution after 10min. This washing was repeated three times.

(iii) Then gel was treated with 0.02% sodium thiosulphate ($Na_2S_2O_3$) for 1min.

(iv) The gel was washed three times in double distilled water (each wash lasting about 20s).

(v) The gel was treated with 0.2% silver nitrate ($AgNO_3$) solution containing 0.075% formaldehyde for 20min.

(vi) The gel was again washed three times with double distilled water (each wash lasting about 20s).

(vii) The gel was developed in 6% sodium carbonate (Na_2CO_3) containing 0.050% formaldehyde and 1 drop of $Na_2S_2O_3$ solution (1mg/mL) until bands appeared.

(viii) The gel was again washed three times with double distilled water (each wash lasting about 60s).

(ix) The gel was placed in a solution containing 50% methanol and 12% acetic acid to arrest further colour development.

3.2.2. ***Protein estimation:*** Protein concentration was estimated as below following the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as the standard:

(i) Preparation of solutions and reagent:

Solution A: Sodium hydroxide (2g) and sodium carbonate (10g) were dissolved in distilled water and the volume was made up to 500mL.

Solution B: It was prepared by mixing 5.0mL of 1.1% of Rochelle salt solution with 0.5mL of 5.5% copper sulphate solution.

Solution C: 50 mL of *solution A* was mixed with 1mL of *Solution B.*

Folin-Ciocalteau reagent: This reagent, purchased from Sisco Research Laboratories Pvt. Ltd., Bombay, was diluted two-fold to obtain 1N immediately before use.

(ii) Procedure: The sample protein in a suitably diluted solution (1.0mL) in a suitable glass tube was precipitated with 0.4mL of trichloroacetic acid (10%) and then centrifuged for 10min at 13,500g. At the end of centrifugation, the supernatant was drained off as completely as possible and the inner wall of the tube was wiped with filter paper. To the precipitate were added 1.0mL water and 5.0mL *Solution C* and mixed thoroughly. After allowing standing for 10min, 0.5mL of *Folin-Ciocalteau reagent* was added with constant stirring and then allowed standing for 1hr. The absorbance of the resultant blue colour was measured against blank (containing 1.0mL of distilled water, 5.0mL *Solution C* and 0.5mL of *Folin-Ciocalteau reagent*) at 546 nm (light path, 1cm). Each batch of the reagent was calibrated with a standard solution of crystalline bovine serum albumin (BSA). A typical calibration plot of the Folin-Ciocalteau reagent for the determination of protein is shown in **Fig.2**

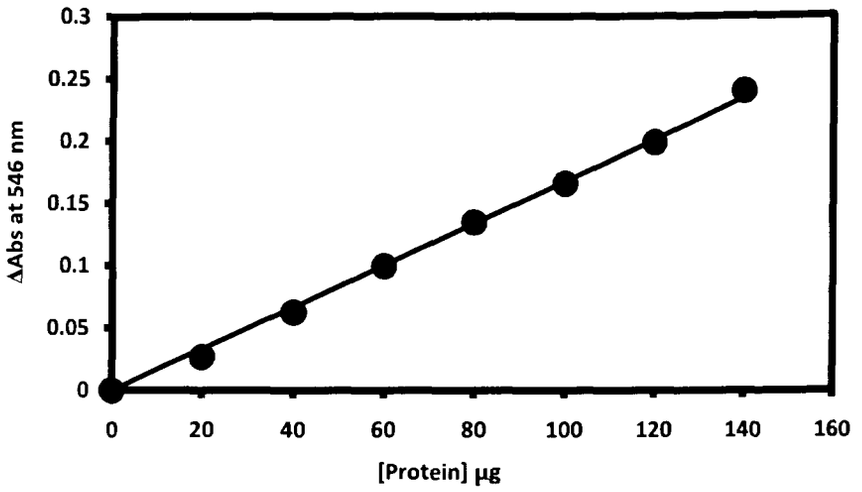

Fig.2: Calibration of Folin-Ciocalteau reagent for determination of protein by the method of Lowry *et al.* (1951)

3.2.3. ***Standardization of carbonic anhydrase assay:*** The following experimental determinations were performed to arrive at the standard carbonic anhydrase assay:

3.2.3.1. *Spectral analysis of p-nitrophenol and determination of its molar extinction coefficient:* A stock solution of 0.01M p-nitrophenol was diluted in 0.5M phosphate buffer pH 7.5 (assay buffer) to obtain a working solution at 10µM. The spectrum of p-nitrophenol in the working solution was determined in the wavelength range 345-555 against the buffer at 30℃. The wavelength of maximum absorption was noted and molar extinction coefficient of p-nitrophenol was estimated based on the absorbance at the wavelength of maximum absorption.

3.2.3.2. *Determination of optimum substrate concentration:* Activity of carbonic anhydrase was determined spectrophotometrically monitoring the esterolytic formation of p-nitrophenol (ϵ_{405}=1.75x10^4 $M^{-1}cm^{-1}$) as a function of the concentration of substrate p-nitrophenyl acetate in an assay mixture constituted by mixing appropriately diluted enzyme sample with the substrate in 0.5M phosphate buffer pH 7.5 at 30℃. The reaction was started with the addition of substrate. After the lapse of 15s of the substrate addition, absorbance at 405nm was noted for every 30s. The corresponding absorbance increase due to slow auto-esterolytic cleavage of the substrate was always deducted to obtain the net enzymatic absorbance increase.

3.2.3.3. *Standard carbonic anhydrase assay:* Carbonic anhydrase was assayed by a standard method which was a modification of the method described by Erel and Avci (2002). The enzyme assay was carried out by spectrophotometrically monitoring the esterolytic formation of p-nitrophenol (ϵ_{405}=1.75x$10^4 M^{-1}cm^{-1}$) in an assay mixture constituted by mixing appropriately diluted enzyme sample with 0.8mM p-nitrophenyl acetate as the substrate in 0.5M phosphate buffer pH 7.5 at 30℃. The reaction was started with the addition of substrate. After the lapse of 15s of the substrate addition, absorbance at 405nm was noted for every minute. The absorbance increase due to slow auto-esterolytic cleavage of the substrate was

always deducted to obtain the net enzymatic absorbance increase. The unit of the enzyme activity was defined as the amount of carbonic anhydrase which catalyzed the formation of one µmole of p-nitrophenol in one minute under the experimental conditions. Protein was determined by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as the standard.

3.2.4. *Standardization of the method of zinc determination by apocarbonic anhydrase re-activation:*

3.2.4.1. *Preparation of apocarbonic anhydrase:* Apocarbonic anhydrase (apo-CA) was prepared by a modification of the method of Erel and Avci (2002) by dissolving the commercial bovine erythrocyte carbonic anhydrase (30mg) in 3mL of suspension buffer (0.2M phosphate buffer pH 7.4) followed by dialysis against the same buffer containing 0.15M pyridine-2, 6-dicarboxylic acid (chelator) for 12h at 4℃. The external chelator solution was then replaced by ice-cold deionized water and dialysis was continued for 8h with two solvent changes. Finally, the external solvent was replaced with the suspension buffer and dialysis was continued for 4h more. After harvesting the dialyzate, it was tested for its residual activity by the standard carbonic anhydrase assay.

3.2.4.2. *Determination of zinc contents in standard solution and solvents*: The concentration of zinc in the standard zinc solution was determined by Flame Atomic Absorption Spectrometer Model Perkin Elmer 3110 in the Sophisticated Analytical Instrument Facility (SAIF), North-Eastern Hill University, Shillong. The standard zinc solution was diluted appropriately in the assay buffer (0.5M phosphate buffer pH 7.5) before the determination. The assay buffer, suspension buffer (0.2M phosphate buffer pH 7.4), substrate solution and water samples purified through TKA Smart2Pure UV/UF (Germany) were checked for their background zinc content by the atomic absorption spectrometric method.

3.2.4.3. *Re-activation of apocarbonic anhydrase:* The apocarbonic anhydrase was re-activated by adding increasing concentration of external Zn^{2+} (zinc nitrate) to the standard carbonic anhydrase assay mixture. The activity restored was assayed by spectrophotometrically monitoring the formation of p-nitrophenol (ϵ_{405}=1.75x10^4 $M^{-1}cm^{-1}$) in the assay mixture constituted by mixing an appropriate amount of apocarbonic anhydrase, externally added Zn^{2+} at different designated concentrations, and p-nitrophenyl acetate as the substrate at 0.8mM in 0.5M phosphate buffer pH 7.5 at 30℃. The reaction was started with the addition of substrate after a lapse of 45s of the mixing of apoenzyme with Zn^{2+}. After the lapse of 15s of the substrate addition, absorbance at 405nm was noted for every minute. The absorbance

increase due to slow autocatalytic cleavage of the substrate was always deducted to obtain the net enzymatic absorbance increase. Determination of activity restored to apocarbonic anhydrase at each designated Zn^{2+} concentration was repeated thrice and each experimental value was obtained by averaging the three corresponding rates of net absorbance change.

3.2.4.4. *Analysis of the apocarbonic anhydrase re-activation data for calculation of zinc concentration in an unknown sample:* The data of activities restored to the apocarbonic anhydrase as a function of Zn^{2+} concentration in the standard assay mixture were analyzed kinetically. The resulting rate equation corresponding to the restoration of activity was used to calculate the unknown Zn^{2+} concentration corresponding to the experimentally determined activity restored to the apoenzyme on addition of the zinc sample. The values of the kinetic parameters (V_{max} and K_m), used in the determination of zinc level, were re-determined and checked every time fresh apocarbonic anhydrase preparation was made.

3.2.5. ***Study on the variation of serum zinc level in psoriasis patients:*** The following procedural steps were followed to determine the nature of variation of serum zinc level in psoriasis patients:

3.2.5.1. *Diagnosis and selection of psoriasis patients:* Patients attending the Department of Dermatology, Regional Institute of Medical Sciences Hospital, Government of India, Imphal during the period January 2007 to December 2009 were recruited. The diagnosis of psoriasis patients and assessment of other clinical parameters were carried out by a qualified dermatologist in the medical institute. A detailed cutaneous and systemic examination was done in all patients. Patients with other skin disorders or systemic diseases were excluded from the study group. Psoriasis was graded clinically by assessing the body surface area (BSA) involved: mild (<5% BSA), moderate (5-10% BSA), and severe (>10% BSA), and lesion-wise consideration of erythema, induration and scaling. One hundred psoriasis patients (n=100) were taken as the study cases.

3.2.5.2. *Selection of control individuals:* The control group comprised of healthy volunteers (n=50) having neither significant medical illness nor undergoing medical treatment for at least 3-months duration at the time of blood collection.

3.2.5.3. *Resolution of ethical issues:* Ethical approval for collecting blood samples of psoriasis patients and control was obtained from the Research Ethical Committee of Regional Institute of Medical Sciences Hospital, Government of India, Imphal. The

patients/guardians and normal individuals were thoroughly informed and their written consents were taken before taking the blood samples.

3.2.5.4. *Serum sample preparation and storage:* Fasting venous blood (3mL) was collected from each patient/control in an AcCuvet Clot Activator tube (manufactured by Symbiosis, a division of Quantum Biologicals Pvt. Ltd., Chennai, India), the inner wall of which is coated with micronized silica particles to enhance clot formation. Blood was allowed to clot at room temperature for about 15min and then centrifuged at 1500g for 20min to separate the serum. The supernatant serum was transferred to a separate sterile vial and kept at -20℃ in the deep freezer till the analysis of serum zinc level.

3.2.5.5. *Analysis for serum zinc level:* The serum samples obtained from psoriasis patients and normal individuals were pre-treated according to the method of Erel and Avci (2002) prior to performing determination of zinc level. The frozen serum was first thawed and mixed with equal volume of 5%(v/v) TCA to precipitate serum proteins. The resulting suspension was centrifuged at 1500g for 30min to collect the supernatant as the serum zinc sample. An aliquot (0.01mL) of the sample was analyzed for its Zn^{2+} concentration by the standardized and specific enzymatic method based on the

apocarbonic anhydrase re-activation described under *Re-activation of apocarbonic anhydrase*.

3.2.5.6. *Statistical analysis:* Statistical analysis was performed by using Statistical Package for the Social Sciences, SPSS version 16.0 (SPSS Inc). The Students t-test and ANOVA were used wherever applicable. The normal range of serum zinc level is 11-22 µmol/L (72-144µg/dL) (Haslett *et al*, 2006).