

Cooperative Role of Ubiquitin E2 and E3 in *Streptococcus Mutans* Dental CariesMalla Sudhakar^{1*}
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Abstract

Periodontal disease or periodontitis is a chronic inflammatory disease which involves a group interactions between the pathogen and cellular host response. It is found to be the most prevalent bacterial diseases in humans. Ubiquitination is the mechanism in which the polypeptide ubiquitin is covalently attached to the obsolete proteins and degrades them with the help of three enzymes namely E1 (ubiquitin-activating enzyme), E2 (Ubiquitin-conjugating enzyme), and E3 (Ubiquitin ligase). Any misfolding or malfunction of these proteins results in the overall instability leading to metabolic disorders in humans. It was studied that some E3 enzymes help in activating the E2 enzymes. The study was aimed mainly to see the role of E2 in the ubiquitination process and to screen the expression levels in the absence of E3. In this study the protein of interest was extracted from the recombinant bacterial clones and purified using the affinity, ion exchange and gel exclusion chromatography. The protein was then studied for the ubiquitination and deubiquitination assays and confirmation was done using SDS PAGE. E3 is normally required for the substrate complex formation, but in contrast we found that in the absence of E3, conjugating activity was found. Our results showed that E2 alone was responsible for the polyubiquitination which was confirmed on the western blot in both the assays. This peculiar feature of E2 can unravel many questions of genetic disorders too in humans.

Keywords

Ubiquitins; E1; E2; Periodontitis; SDS PAGE.

Introduction

Periodontal disease often called as periodontitis is a chronic inflammation initiated by the bacterial infection that eventually leads to destruction of the gingival tissue and alveolar bone resorption. The dental tissue's response to the plaques is mostly characterized by the accumulation of polymorphonuclear leukocytes, macrophages, and lymphocytes, where they initiate to release the inflammatory mediators to regulate the pathogenesis of the disease [1]. Ubiquitination is a mechanism which involves a number of factors, including an ubiquitin-activating enzyme, ubiquitin-conjugating enzyme, and ubiquitin-protein ligase all of which are involved in essential host processes. The ubiquitin system was found to be the sole responsible for the immune response, progression and events of programmed cell death seen in periodontitis. Several studies did so far provide a basic evidence of the relation between ubiquitination and the disease and its progression. A recent study done on the polyubiquitinated proteins proved of its essential role in accumulation on the surface of bacteria [2]. Cox et al., (1986) confirmed of the role of the ubiquitin system in recognition of the bacteria in the mammalian cytosol [3]. Kieffer et al., (2003) confirmed of the active role of ubiquitin N- and C-terminal-derived peptides in antimicrobial activity [1].

Ubiquitin is a very small protein which exists in all the eukaryotic cells and functions by conjugating to large number of target or obsolete proteins. In simple words Ubiquitin helps to tag all those obsolete proteins for destruction. It consists of about 76 amino acids and is approximately 8.5kDa in molecular size. It has its usual C-terminal end with 7 lysine residues and is highly conserved among all the eukaryotic species. Several modifications occur within the protein for effective functioning on the targets. They are a set of proteins which display similar structures, but exist with variable sequences [4]. Prokaryotes do not possess these types of proteins which attach to another protein by covalent attachment. But instead they do contain proteins which seem to be the ancestors of ubiquitin. ThiS and MoaD are the bacterial proteins which aid in the insertion of sulfur into the thiamin and molybdopterin, respectively. The protein confirmations are very much similar to that of ubiquitin.

Cells build up the proteins (structural or physiological) in a continuous process. Most of these proteins are used for the process and discarded when not in use. This sort of homeostasis is maintained by the cell machinery. Signalling proteins like transcription regulators, and cyclins which control the process of cell division have very short half lives as such they need to be recycled back immediately [5]. Even in the case of some specialized

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enzymes, cells build them and discard them immediately after use. Such a system would be a mere waste but it is very much needed to keep in pace with the minute by minute activities of the cell. But such destruction of the obsolete proteins will be taken care of by the cell machinery with the help of small proteins. Then comes ubiquitin into picture. Ubiquitin comes and attaches to all the proteins which need recycling, and signals them to the cell saying they are ready for disassembly. A set of ubiquitin proteins then comes and attaches to the string and are recognised by the destruction machinery. The major role played by the ubiquitins lies in their tricky nature of destruction. They make sure that they attach to the obsolete proteins only and as such they need specialized enzymes for the action. These enzymes are of three types namely E1, E2, and E3. E1 is the ubiquitin-activating enzyme which aids in the initiation of the process. An ATP is used by the enzyme, in this process. E1 then transfers the activated ubiquitin to E2 enzyme which is called as the conjugating enzyme [6]. These E2 enzymes work in coordination with a number of enzymes called E3 (which are large in size) to recognize the waste proteins. The E3 enzyme cluster shapes up like a clamp and the target protein now binds in the gap.

Once these obsolete proteins are tagged with a minimum of four ubiquitin molecules, they are now ready for destruction by proteasomes. Proteasomes are commonly called as protein shredders which shape up like a cylinder. All the active sites are arranged in the interior of the cylinder [7]. The cap like structures which are present at the ends control the entry of the proteins where they are chopped into small pieces of 3-23 amino acids long. Ubiquitination is the mechanism in which the polypeptide ubiquitin is covalently attached to its target proteins. It is one of the post translational modification carried out by three enzymes namely E1 (ubiquitin-activating enzyme), E2 (Ubiquitin-conjugating enzyme), and E3 (Ubiquitin ligase). This mechanism leads to change in the overall stability of the protein and its biological activity [8]. Any mutation or change in the overall stability of the ubiquitination pathways leads to metabolic disorders in humans [9]. Most of the diseases like cancer, neuronal dysfunction, and metabolic disorders are all found to be linked to this altered mechanism. Study of these ubiquitin proteins or any alterations made to these proteins can be of specific interest as it throws light on the pathogenesis of the diseases.

Our understanding of the diseases in humans in relation to the ubiquitins has expanded over the years. Even the link between the signalling pathways and the proteosome degradation has been established long back. But still studies done on the individual enzymes are very less. As such more confirmatory studies involving the class of Ubiquitin proteins is very much essential to down regulate the diseases in humans. This would lead to the development of markers for the disease diagnosis and also show light on the probable mode of treatments.

In this experiment, the protein of interest E2 will be studied for its conjugating activity in the absence of E3, a ligase enzyme. As part of the study bacterial protein will be extracted from the cells which already been induced for the E2 expression. The protein of interest was extracted and purified using the affinity, ion exchange and gel exclusion chromatography. The protein sample thus obtained was used for ubiquitination and deubiquitination assays to confirm of the role of E2 in enzyme activity. SDS PAGE and western blot was performed to confirm the activity of the E2 (conjugating enzyme).

Materials and methods

Revival of culture

Streptococcus mutans was procured from MTCC (Cat No: 497) Chandigarh, India and revived using the instructions provided. Brain heart infusion agar was used for reviving and the colonies obtained were subcultured for further use.

Protein Expression on small scale

To 5ml of LB broth (sterile) 5μL of Kanamycin (50mg/mL) was added and a loopful of inoculating culture was inoculated and incubated at 37°C shaker/incubator for overnight. 25ml of LB media

was added with the respective antibiotic and inoculated with 250μL (1:100) of the starter culture prepared earlier. The culture was incubated at 37°C shaker/incubator for overnight. The overnight culture was used as starter culture for the next day.

Determination of Bacterial growth

The bacterial growth was determined using the spectrophotometer. Briefly, 1ml of sample was taken in 1.5ml of cuvette and the optical density at 600nm (OD₆₀₀) was recorded. Sterile LB media was used as control. OD values were recorded before and after induction with IPTG (0.5mM). Induction was carried only to the culture at 0.6-0.8. The flasks were incubated in a shaker/incubator at 20°C overnight. The contents were collected and centrifuged at 3200xg for 20min in a cooling centrifuge and the supernatant was discarded and pellets were stored on ice.

Protein isolation

The pellets were resuspended in protein extraction reagent (50mM Tris-HCl, 300mM NaCl, 20mM Imidazole, 0.05% β-mercaptoethanol, 0.5% triton X-100). The extraction buffer was maintained at pH8.0 and filter sterilized with 0.22μm filter and added with 1mM PMSE, phenylmethylsulfonyl fluoride just before use. The contents are mixed properly and incubated on roller for about 10-20min at room temperature. Following incubation the contents were collected and centrifuged at 4°C for 20min at maximum speed. The supernatant was collected and labelled accordingly and stored for SDS PAGE.

Bacterial protein extraction

The bacterial strains (*E.coli* DH5α) were transformed with pRSF_T6 Ube2t, using kanamycin (50μg/mL) as the natural marker. The protein of interest cloned was MCS1 which is His6-Ube2t (His tagged). The protein sequence of interest which was cloned into the plasmid was given below.

MHHHHHHSHENLYFQGSMQRASRLKRELHMLATEPPPGITCWQD
KDQMDDLRAQILGGANTPYEKGVFVKLEVIIPERYPFEPPIRFLTPY
HPNIDSAGRICLDVLKLPKGAWRPSLIATVLTSLQLLMSEPNPDDPL
WTEKHARQKQKADEEEMLDNLPEAGDSRVHNSTQKRKASQLVGI
EKKFHDPV

The molecular mass of the given sequence was calculated using the online calculator tool (<http://protecalc.sourceforge.net/>). The molecular mass was found to be 24638.0410. The bacterial pellet collected was thawed and added with 1X EDTA-free protease inhibitor followed by 1mL of buffer E-A. The contents were mixed thoroughly and incubated for 5min at 37°C before vortexing. The contents were now added to defrosted bacterial pellet lysed with 1mL of lysozyme (30mg/mL) solution and 3μL Nuclease stock solution (200units/μL). The contents were incubated on a tube roller initially for 30min at room temperature and then 30min on a tube roller at 4°C. A 5μL aliquot sample was removed and placed in microfuge tubes and were stored in the freezer until further use.

Determination of protein concentration

The supernatant collected was subjected to estimation of concentration. The concentration of the protein sample was calculated directly by UV absorption at 280nm. 1ml of supernatant was added to 1.5ml cuvette and placed in the UV spectrophotometer and observed for the optical density at 280nm. Readings are noted at both 260nm and 280nm. Sterile distilled water was used as the blank. The readings were tabulated and calculated using the formula.

SDS PAGE

To study the protein profile unidirectional SDS-PAGE was performed on 10% separating gel and 5% stacking gel. This was done in a mini vertical gel system. About 100μg of protein sample was loaded in each sample well. 10μl of sample loading buffer with bromophenol blue was added as the tracking dye. The first well was used for protein ladder. The gels were run at a voltage of about 100V for 3hrs. Following the run, the gels were placed in tray containing coomassie brilliant blue and incubated on the shaker or roller for overnight. The gel was photographed and stored for further use.

Ion exchange chromatography

An ion-chromatography resin which binds a positively charged species is known as a cation exchanger. Most of the proteins which are negatively charged or neutral at pH 7 will not bind to the resin. As such we need to use a commercial cation exchanger like a 1mL SP Sepharose (HiMedia). E2 is usually positively charged at pH 7 from its isoelectric point. 1mL SP Sepharose fast flow column was used with 200mL Buffer E-C (50mM Hepes pH 7 / 5% glycerol / 1mM DTT). The column was equilibrated with buffer A (50mM Hepes pH 7 / 150mM NaCl / 1mM DTT / 5% (v/v) glycerol) for 4-5 hours. The samples were eluted with 100mL Buffer B (50mM Hepes pH 7 / 1000mM NaCl / 5% glycerol / 1mM DTT). All the fractions were pooled from the affinity step into a 50mL tube. Only the purest fractions which contain proteins were collected and an aliquot of 40µL was removed and stored.

Ubiquitination Assay

Ubiquitin a small signalling molecule present ubiquitously in the cellular locations. As such it is called ubiquitin. It is the most versatile signalling molecule as it can be linked to chains. The cascade is a 3 step process which requires three enzymes for priming, transporting and linking the ubiquitin molecules to the target protein. E2 protein is specialised protein for activation of pathogenesis in periodontitis disease. It aids in repairing the interstrand crosslinks (ICL's) in DNA. In the absence of this function, the cells become susceptible for carcinogenic affects. This is due to the close vicinity of a Lysine residue to the cysteine through other sites. Three different proteins namely E1, E2 and ubiquitin are mixed in the presence of ATP and incubated at 37°C. The samples treated are then analysed on SDS gel for further validation. To tubes labelled as +ATP (with ATP) and the second -ATP without ATP are added with the specified reagents in the order as shown in the Table 1. At time T=0 a 10µL aliquot was removed from the tubes and the reaction was stopped by adding 3.3µL of gel loading buffer. The two samples were labelled accordingly and the process was repeated at 30min and 60min of time. Finally the aliquot at 60min was added with 1µL of apyrase (0.5units/µL) to both the reaction tubes. The reaction mixtures were freezeed and used for deubiquitination assay. The samples obtained were run on SDS PAGE for further validation along with the protein ladder.

Deubiquitination Assay

The assay was carried basing on the result analysis of the ubiquitination assay. Sample of ubiquitinated protein, DUB solution, 4X SDS-PAGE loading buffer with β-mercaptoethanol and SDS-PAGE gel apparatus are required for the experiment.

About 45µL sample (+ATP run) from the ubiquitination step was defrosted and a 10µL of aliquot was added to 3.3µL of SDS-PAGE loading buffer and stored at room temperature. To the contents 1µL of DUB (0.5µM) was added and incubated at room temperature for 15 min. to the tube add 3.3µL of SDS-PAGE loading buffer and the reaction was stopped. The sample tube was labelled accordingly and the experiment repeated at 30min. The two samples obtained (15min, 30min) were run on SDS-PAGE gel along with the protein ladder. Sample without DUB serves as the control.

Results

The sample proteins obtained were estimated for the concentration at 260nm and 280nm. The concentration of the sample from the given spectrophotometer report was found to be 0.01647.

	+ATP assay	-ATP assay
Buffer	54 µL	57 µL
MgCl ₂	3 µL	3 µL
E ₂	9 µL	9 µL
E ₁	3 µL	3 µL
Ubiquitin	3 µL	3 µL
ATP	3 µL	0 µL

Table 1: Reagent volumes used in Ubiquitination Assay

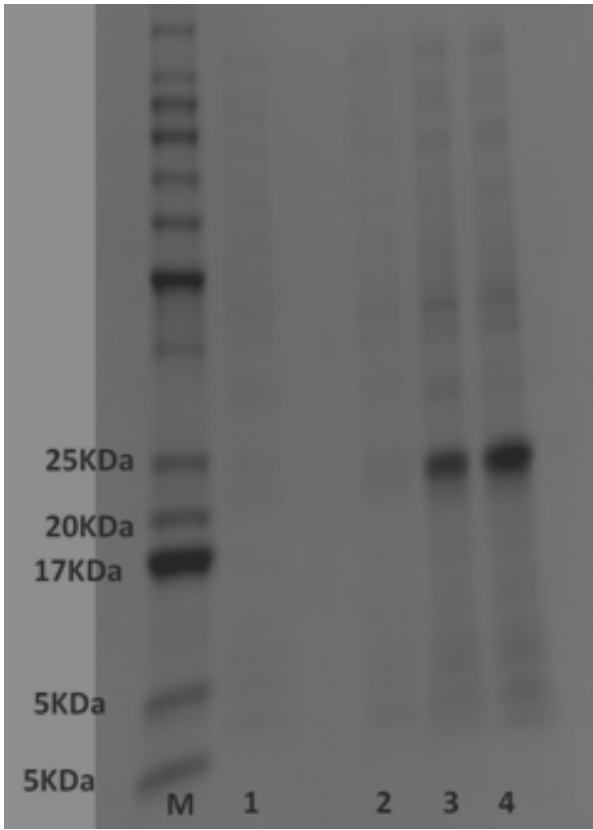


Figure 1: SDS-PAGE of the expression proteins. Lane1: Ladder; Lane 2 & 3: Before induction; Lane 4 & 5: After induction.

The absorbance at 280nm and 260nm was found to be 0.013 and 0.017 respectively.

SDS-PAGE of Small Scale Protein Expression

The SDS PAGE gel of the proteins before and after induction showed significant results. Before induction, in both the whole cells and cell extracts, there was no significant protein expression. On induction, there was an ample amount of protein expression observed. This proves of the positive induction which expressed the desired protein. The bands were found to be approximately of 24kDa from figure 1. Both lane 4 and 5 showed expression of the protein. The protein was found to be expressed after induction with the IPTG. Lane 2 and 3 showed no expression.

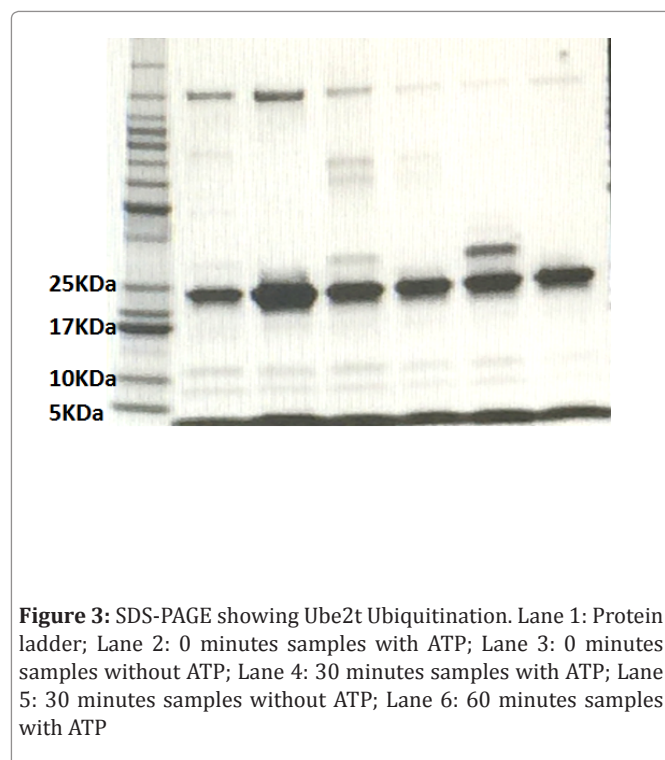
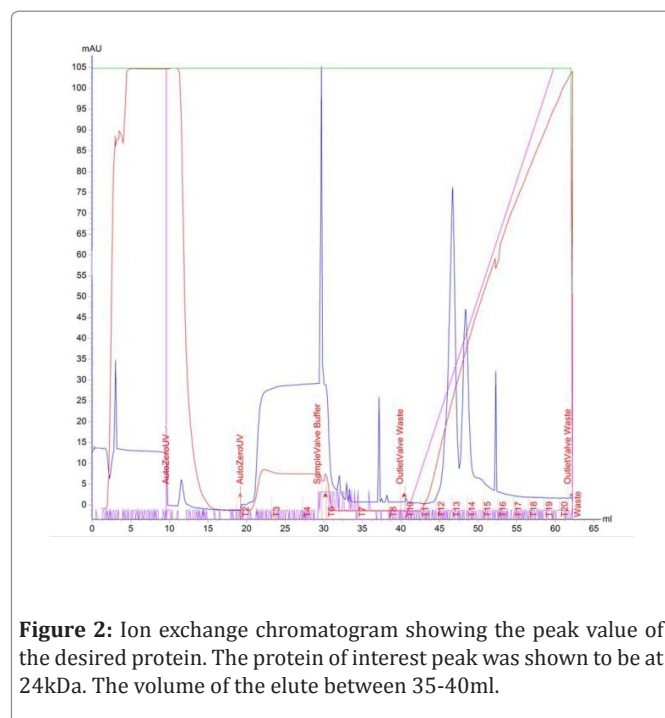
ION exchange chromatography:

Ion exchange chromatography was done to purify the protein of interest. The protein of interest was E2 which was found to be expressed in the bacterial cells (*E.coli* DH5α) using the plasmid pRSF_T6 Ube2t. The protein of interest cloned was MCS1 which is His6-Ube2t (His tagged).

On separation of protein fractionation, the supernatant was allowed for ion exchange to elute the protein of interest. The protein of interest was found to be approximately around 24kDa. As shown in the figure 2, the protein of interest was found at a peak of molecular weight 24kDa between the 35-40ml of elute volume. The sample component was collected and later used for the SDS PAGE analysis for validation.

The samples run on SDS PAGE showed that the fractions are pure in nature

The protein band of interest was found to be approximately between 25 and 23kDa (data not shown) which was correlating to the hypothetical calculated value (24638.0410) from the online calculator. The protein bands confirm of the presence of the desired protein. All the samples were pooled and used for the Ubiquitination assay.



Ubiquitination

The E2 conjugating enzyme tested in the reaction catalysed the substrate ubiquitylation assay. From the data it seems likely that E2 conjugation with the substrate was possible at 30min and thereafter. In lane 2, the substrate was free in nature, whereas in lane 4 the substrate conjugation was seen clearly. This clearly indicates the role of E2 in conjugation. As from the figure 3, the conjugation increased with the time. In lane 6 the conjugation of E2 can be clearly depicted.

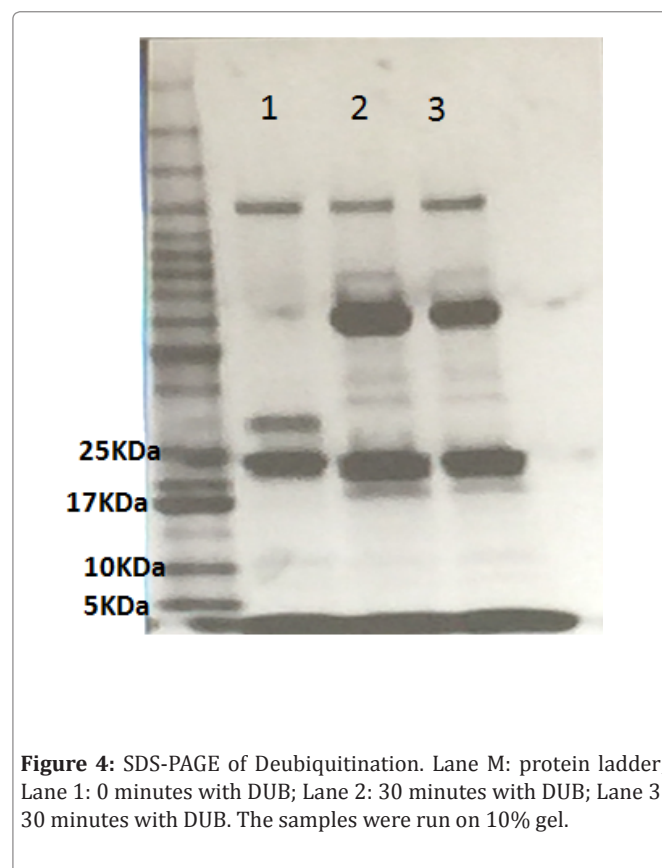
Deubiquitination

From the figure 4, it is clearly understood that the E2 conjugation was decreased as the time increased. In lane 1, the conjugated sample was found to be deconjugated. In lane 3 and 4 the deconjugated substrate can be seen away from the E2. This clearly depicts of the role of E2 in conjugation.

In the figure 4, E2 protein was observed at about 24kDa. Once DUB was added initially, the deconjugation of the protein can be seen clearly. In lane 3 and 4, the deconjugated substrate was found to be separated at about 50kDa.

Discussion

Ubiquitination is a post-translational modification which is reversible in nature and extensively used during regulation. The attachment of ubiquitin to some of the cellular proteins influences many such pathways and in turn disrupts the protein localization and proteasome degradation. This regulation is linked to many diseases in humans like cancer, neuronal dysfunction and muscle wasting diseases. The E2 conjugating enzyme can either directly bind to E3 or directly transfer the ubiquitin to the target protein. In order to carry



this function, E2 binds to E1 and activated ubiquitin. Humans possess about 35 different types of E2 enzymes which are highly characterised and highly conserved in nature. The results obtained were found to be successful. The proteins isolated were quantified using the UV spectrophotometry and was found to be pure. The concentration of the protein sample was found to be promising for the filtration techniques. From the figure 3, it was observed that the band was our protein of interest with an approximate molecular weight of 23-24kDa. The sample obtained was confirmed of the E2. This sample was further purified on gel exclusion chromatography. The elutes were pooled for the total fraction and used for the ubiquitination assay and deubiquitination assay.

Conclusion

The main approach of the study was to confirm of the assertive role of E2 in autoubiquitination. As the experiment was done in the absence of E3, the role of E2 was possible defined and understood. The data clearly shows the role of the E2 in directing the ubiquitination. From the results, it was understood that the E2 aids in ubiquitination. And from the present study our findings confirm the possible individual role of E2 in conjugation. As E2 is thought to act lonely or in conjunction with E1, the activity was mainly to confirm the role of E2. Hence as the study was without E2 and the results

also shows of its nature. Further experiments need to be planned on ELISA which shows specific activity. Antibodies can be designed for the E2 and E3 and can be carried using the sandwich mode of ELISA. Further it can also be confirmed on the knock out cell lines. Using RNAi technology, specific E3 knockouts can be created and used for the experimentation. Studies on knockouts confirm the findings not only at biochemical level but also at the molecular level. On summing up, the E2 protein can be studied in detail about its mechanism and its action. This can unravel many questions of genetic disorders in humans. This peculiar feature of E2 conjugating enzyme to complete the cascade in the absence of E3 would surely be of interest for the scientific field. Moreover, we can expect a better understanding of the prognosis of the diseases and in developing diagnostic tools.

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