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International Journal of Current Research
Vol. 9, Issue, 07, pp.54365-54368, July, 2017

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

EFFECT OF DNFB TREATMENT IN COMBINATION WITH DIFFERENT PHS OF THE MEDIUM ALONG THE LENGTH OF *VORTICELLA* STALK CONTRACTION DYNAMICS

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ARTICLE INFO

Article History:
Received 16th April, 2017
Received in revised form
25th May, 2017
Accepted 13th June, 2017
Published online 31st July, 2017

Key words:

DNFB, protonation, *Vorticella* stalk, frequency.

ABSTRACT

Vorticella stalk contractility is a non-Newtonian type movement exhibiting positive DNFB (Sanger's reagent or 1-fluoro-2,4-dinitrobenzene) sensitivity when in acidic medium. This brings 25 to 30% change in contraction frequency with 0.5 to 1 fold variation in electrochemical gradients. Positive effect towards protonation supports the protoosmotic conduction (Verma AK and Singh AK, 2017) and pin-points towards reactivation of binding sites of *Sfi1p* and *Cdc31p* binding ryanodine receptors. *In vitro* applications of spasmin protein through controlled activation sites of functional terminal domains of amino acid residues and getting aligned with binding receptor sites indicates about its possible multiple applications.

Citation: Amit Kumar Verma and Ashok Kumar Singh, 2017. "Effect of DNFB treatment in combination with different PHS of the medium along the length of *Vorticella* stalk contraction dynamics", *International Journal of Current Research*, 9, (07), 54365-54368.

INTRODUCTION

The stalks of all vorticellids have been regarded as a model system in the field of physiological and biochemical investigations. The amino acid residues of spasmin protein (spasmoneme of the stalk) are tyrosine, cysteine and histidine as investigated in glycerinated stalk (Sokolovsky et al., 1996). Spasmin protein has homology with *EF*-hand domain of centrin (32%) and calmodulin (25%) which rapidly contracts on Ca^{++} sequestration. These proteins have multiple repeats of *Sfi1p* and *Cdc31p*. Their stretching is brought about due to unfolding of tandem repeats (Tatham and Shewry, 2000). *Sfi1p* is a very divergent polypeptide (120-kDa with 21 motifs) with 24.5% sequence similarity with spindle pole body (SPB). It has a series of striking internal repeats ($AX_7LLX_3F/LX_2WK/R$) conserved in other species with possible exception of repeats around W304 and F698. Sometimes alanine is not prominent. The gap between clusters of repeats is of about 23 to 35 amino acids. The expression of 15 repeats of *Sfi1p* leads to formation of filaments of *F-actin*. This protein has repeats similar with *Sfi1p* of *Schizosaccharomyces pombe* (T40750) and a human protein (KIAA0542) which are respectively called *SpSfi1* and *hSfi1*. There are at least two versions of *hSfi1* with almost identical sequence. Ca^{++} has no effect on the length of filaments (Li et al., 2006). DNFB is well known hexagonal (benzene ring) that acts as metabolic uncoupler. Its reactive end $2NO_2^-$ (2,4-dinitro terminal functional group) combines with NH_2^+ terminal group of amino acid residues, resulting into the formation of dinitrophenyl (DNP) [$N_3O_2H{-}COOH^-$ or $(NO_2^-)_2NH{-}COOH^-$] derivatives in hydrolytic reaction. This remains moderately stable in activation medium. With increasing concentration of DNP, neutralization of NH_2^+ of amino acid residues increases. This by means of inhibitory effect biochemically knocks out *Lohmann reactions* in skeletal muscle at room temperature (Raj *et al.,* 1989). On the other hand fluoride terminus (1-flouro) changes colligative properties of the surrounding medium and brings variations in the rate of reactions concerned with contraction-extension cyclic phenomena. Thus the experiments to investigate the effects of DNFB on the *Vorticella* stalk contraction process has been undertaken which could be correlated with the folding and unfolding of the above explained active sites of spasmin/centrin proteins which may have direct manifestation on the contraction of stalk.

MATERIALS AND METHODS

The frequency and duration of stalk contractility of vorticellids were recorded under known conditions of different DNFB concentrations of solutions. Before experimentation, the specimens of *Vorticella* were kept in standard controlled medium for 24 hours. The culture of *Vorticella* was used to be transferred into its sub-culture after every 2 days. Different DNFB concentrations of 1mM, 2mM, 3mM, 4mM and 5mM were used for the experiments. Under controlled bathing media

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(pH 7.0) of different DNFB concentrations, the washed specimens of *Vorticella* were kept immersed for approximately 1 minute into the solution containing 20 mM KCl, 10 mM EDTA and 10 mM of Tris-maleate buffer. The extracted specimens were then washed three times with a washing medium composed of 50 mM KCl, 2 mM EDTA and with 10 mM Tris-maleate buffers for 15 minutes. The extracted specimens were kept immersed into the reactivation media of different H^+ concentration (from 1mM to 5 mM). The reactivation media were consisted of 50 mM KCl, 10 mM Tris-maleate buffer and H^+ buffer solutions. The experiments were performed at room temperature to observe the complete contraction-extension cycles. The lengths of stalks of many *Vorticellids* were measured by linear distances from the base of the stalks i.e. from their attachment point with substratum to the bell/zooids. Data (i.e. video recording) transferred to the computer was recorded on paper then it was bio-statistically measured by using the formula of mean and standard deviation. The data obtained have been represented graphically. It is assumed that in these cases, n moles of H^+ bind to the contractile elements in the contracted state and the binding brings about contraction of the contractile elements which can be represented by following equation (Ochiai et al., 1979):

$$[S] + [H^+] \leftarrow K \rightarrow [S.H^+_n]$$

Where, [S] is the element of contraction whereas $[H^+]$ is the factor bringing contraction by reducing expense of energy.

RESULTS AND DISCUSSION

As our idea is to find the evidences to explain the process of contractility in *Vorticella* stalk on the basis of protoosmotic model the change in pH conditions was taken as important parameter.

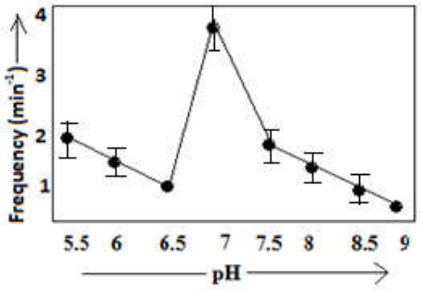

Fig .1. Effect of different concentrations of DNFB in combination with different pHs representing average frequencies during spontaneous contractions

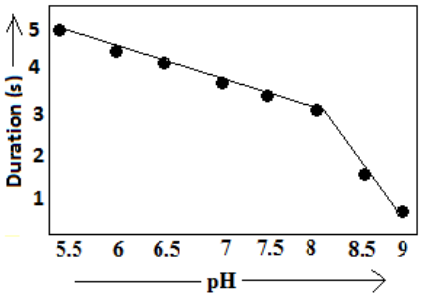

Fig. 2. Effect of different concentrations of DNFB in combination with different pHs representing average duration during spontaneous contractions

This can generate the proton gradient in the medium. Surprisingly at pH 7.0 the effect of all concentrations of DNFB was found to be detrimental and the frequency of contractility was gradually reduced which means the DNFB is having poisonous effect at normal pH. But, the contractility improved when lower pHs i.e. 5.5, 6.0, 6.5 was introduced. Here the role of DNFB is positive which perhaps ameliorate the adverse effects of high H^+ concentration in the medium.

Table 1. Effect of DNFB treatment in combination with different pHs of the medium along the length of *Vorticella* stalk contraction dynamics where S.D. = ±0.02 and N = 4

[DNFB] + pH	F/minute	MP in mV	Amp in mV	HW in mV	PPT in mV	SPR in mV
5.5	2.0	3.5	8.9	50.6	0.96	19.82
6.0	1.5	2.6	6.7	38.1	0.72	14.86
6.5	1.0	1.7	4.4	25.4	0.48	9.91
7.0	3.5	6.2	15.9	97.5	1.68	34.96
7.5	2.0	3.5	8.9	50.6	0.96	19.82
8.0	1.5	2.6	6.7	38.1	0.72	14.86
8.5	1.0	1.7	4.4	25.4	0.48	9.91
9.0	0.5	0.8	2.2	12.7	0.24	4.95

The effects of different concentration of DNFB have almost the same all the above shown different pHs. This shows that only the few molecules of DNFB are required to generate and exhibit the effect and the rest surplus molecules (as at its higher concentrations) have no significance whether present in the medium or not. In this biochemical and physiological reactions, DNFB directly affects the orders of reaction kinetics at acidic pHs which affects normal proton motive force production and its relation to the folding kinetics of ryanodine receptors. At acidic pHs ranges from 5.5 to 6.5, DNFB reduces acceleration potentials of motive force production. Here DNFB supports inertial force (in Bernouli's formula) rather than viscous force (in Stoke's formula). It resembles the behaviour of damped harmonic oscillation in relation with equation of motion. It is not in favour of third law of motion but instead supports second law of thermodynamics and thus is non-Newtonian in characteristics by the law (equation) of motion applied to the hydrodynamics to continue hydromechanical performances of the stalk (protoosmotic cable conduction).

The time-lapse images of contraction dynamics (Fig. 2) indicates that the DNFB at acidic pH increases the duration of torsion-detorsion process of zooid along with duration of contraction-extension cycle of the stalk. This may be due to reducing the normal membrane potential and the action-potential along the length of the stalk at both ends as result of acidic pH ranging from 5.5 to 6.5. It also affects the amplitude and half-width of the action potentials of the stalk along the length from base to *scopular region* (Shiono and Naitoh, 1997). There is no *Lohmann reaction* occurring here as in the case of skeletal muscle. It is clear from the basic mechanics of stalk contraction-extension cycles of past and present works.

It supports energy transduction processes and conformational changes of *F-actin*, independent of ATP hydrolysis, molecular crystallization and rigor mortis at this acidic level. The physiological and biochemical properties of the stalk and the medium oppose similarities between the molecular characterisations of the actomyosin and spasmin based bio-chemo-mechanical nature of these two varieties of proteins involved in cell motility.

The reaction rate between DNFB and tyrosine increases with increasing acidity of the experimental media. Nitration does not occur at acidic pH 5.5 to 6.5 below 7.0. At these pHs

degree of contractility decreases by 20 to 40% in combination with [DNFB] from 1 mM to 4 mM and is reversible at room temperature. The resistance of [DNFB] has no significant role on contraction dynamics of the stalk in the range of alkaline medium pH 7.0 to 9.0 (Riordan et al. 1996). It indicates that DNFB has more pronounced effect at acidic medium and only few molecules try to prevent reaction-kinetics. They can also oxidize sulfhydryl group of cysteine residues at defined pHs range. This experiment shows that at low pH condition, the effect of DNFB is ameliorated and protons establish protic cable connection. Thus instead of impairing mechanical performances, it improves contraction dynamics of the stalk. At low pH, thus the role of Ca^{++} is possibly played by H^{+}. Thus this protoosmotic theory clarifies that the performances of the stalk fibre (*spasmin*) and the bell (*myoneme*) depend upon the proton motive force production processes at low pH and not at ATP hydrolysis which is paid by mechanical performances of the micromachine. This principle can be used to control hydrophobic patches of the spasmin protein through controlling ryanodine receptor which will be advantageous in controlling physiological as well as biochemical performances of spasmin proteins in vitro or in vivo. This may be applicable in other systems also. In this reaction kinetic mechanism, DNFB molecule (D) binds with ryanodine receptor (R) of spasmin protein represent hyposensitivity and low affinity to that molecule which can be denoted as:

$$D + R \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} D\text{–}R$$

Where, K_1 represents forward association rate constant whereas K_{-1} represents reverse dissociation rate constant. In this reaction kinetics, the ratio K_1/K_{-1} is representing association constant K_a with the measured affinity due to its equilibrium constant for the above equation which can be calculated from the ratio of molar concentration of above bound D−R complex to the molar concentration of unbound DNFB and ryanodine receptor of that molecule. At equilibrium constant, it can be derived as:

$$K_a = \frac{[D\text{–}R]}{[R]\,[D]}$$

Here, the value of Ka varies with differences in D−R complexes which depend upon both K_1 and K_{-1}. The K_1 is expressed in units of liters/mole/second (L/mol/s) whereas K_{-1} into the units of 1/second. In this reaction kinetics, few molecules bind in association with forward rate constant with extremely high rate. In some cases, K_1 is high as 8.9×10^7 L/mol/s approaching its upper limit of diffusion-limited rate reaction which is 1.9×10^8 L/mole/s. For alkaline media, K_1 is smaller with the range of 1.9×10^5 L/mole/s. Overall the mechanisms and the rate at which bound DNFB leaves ryanodine receptors of spasmin protein represents its dissociation rate constant (K_{-1}) which plays a major role in determining DNFB affinity for the ryanodine receptor of the spasmin proteins and their isotypes. As per above kinetics the protoosmotic conductivity, protonation and deprotonation can be extrapolated according to following table. In these biochemical catalytic reactions data indicate that protonation works in association with deprotonation for maintaining equilibrium. In these cases, DNFB rate constant is directly proportional to the *Fenton* and *Heber-weiss* reactions rate constant and is inhibited by protoosmotic conductivity which works on the basis of *Lewis* acid catalytic reaction in response to motive force production. In these interrelated biochemical phenomena, energy exchange is directly associated with the production of redox-potentials which is equivalent to *Nernst* potential (E') or *Henderson-Hasselbatch* equation.

$$E' = E_0' + 0.03 \log \frac{[H^+ \text{ acceptor}]}{[H^+ \text{ donor}]}$$

In these cases electrochemical titration gradient (E_0') can be defined as Ka or pKa through the mid-point of acid titration curve where electrochemical gradient variation is 0.5 to 1 fold due to DNFB treatment. Spasmin binding kinetics works in consonance with folding-kinetics of *Cdc31p* and *Sfi1p* (Gogendeau et al., 2007) where *Sfi1p* and *Cdc31p* get unfolded and combine as per the induced-fit model of enzyme kinetics. The folding and unfolding of these two elements of spasmin proteins continues the contraction-extension cycles of the specimen at rapid rate.

Table 2. Representing protoosmotic conductivity, protonation and deprotonation in sequential enzyme kinetic reactions where S.D. = ±0.02 and N = 4

[DNFB] + pH	$[H^+]$/mole	Protoosmotic conductivity	Protonation (pKa_1)	Deprotonation (pKa_{-1})
5.5	14.80×10^{-10}	12	3.1×10^{11}	6.6×10^4
6.0	12.10×10^{-10}	11	3.0×10^{11}	5.5×10^4
6.5	09.12×10^{-10}	10	2.9×10^{11}	5.0×10^4
7.0	06.14×10^{-9}	09	2.8×10^{11}	4.5×10^5
7.5	03.16×10^{-9}	08	2.7×10^{11}	4.0×10^5
8.0	06.18×10^{-8}	07	2.6×10^{11}	3.5×10^5
8.5	00.14×10^{-8}	06	2.5×10^{11}	3.0×10^6
9.0	00.12×10^{-7}	05	2.4×10^{11}	2.5×10^6

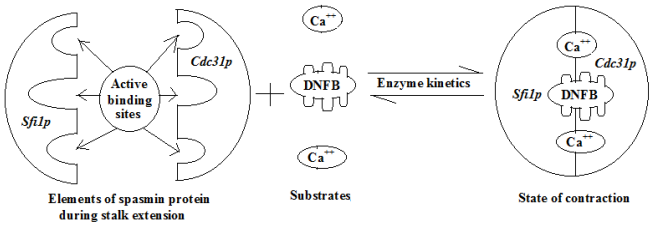

Fig. 3. Induced-fit model of enzyme-kinetics of elements of spasmin protein (*Sfi1p* and *Cdc31p*) with active binding sites (ryanodine receptors) during *Vorticella* stalk contraction dynamics

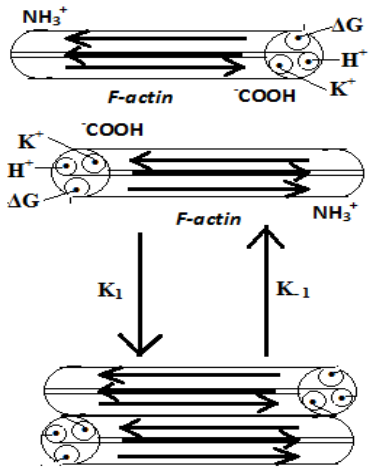

Fig. 4. Protoosmotic model of molecular movement and energy transduction along the length of *F-actin* and in the spasmin protein of *Vorticella* stalk during its contraction dynamics

On the basis of present and past works it is clear that the mode, path and pattern of molecular movements and energy transduction are the same as in muscles, *Vorticella* stalk and neurons. Structural predictions depict differences in planes of surfaces as smooth groove surface for sliding-filament movement of acto-myosin as in skeletal muscles while it is twisted and wounded with helical eccentric bending in spasmoneme. In neurons, intra-cytoplasmic bidirectional molecular movements along the length of *F-actin* is facilitated inside the cytoplasm bounded along inner face of neurilemma.

In all these types of cell motilities the electrochemical and physiological performances are same. The directions of Ca^{++} and H^{+} conduction are same whereas it is opposite for K^{+}. DNFB interference affects energy transduction along the length, path and patterns of cell motility and thus affects its rate constant in overall related biomechanical processes. These all are related with amino acid activation sites (functional groups at the terminus) and their degree of bending patterns of peptide bonds between nearby amino acids which varies with DNFB treatment.

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