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Original Article

Influence of Copper Sulphate on Reaction of Formation of Disulphide Bond in Desmopressin using Combination of Different Solvents

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ABSTRACT

A series of experiments were performed to evaluate the effect of a catalyst (copper sulphate) on the rate of disulphide bond formation of desmopressin. Parameters that were considered during the evaluation were reaction time, yield, chromatographic peak purity and ease of processing during reaction work up. Experiments were done by using solvent as water, 50%Water : 50%acetonitrile, 50%Water:50%methanol and 50%Water:50%ethanol. A concentration of 1 mg/ml of desmopressin was chosen for the experiments. For each solvent pair, a set of two oxidation reactions was studied at concentrations of 1 mg/ml. One set was done without addition of copper sulphate and in a second one adding catalytic amount of copper sulphate at the start of reaction. It was observed that the use of organic solvents has increased the solubility of desmopressin Bis-SH and therefore the yield obtained increased. The use of catalyst increased the rate of the reaction by 50%. The reaction in 50%Water: 50%acetonitrile at 1mg/ml concentration using copper sulphate is the most effective for formation of disulphide bond. The area of desmopressin is 1.5 times more than the initial area of desmopressin Bis-SH and reaction completed was in 3 hours. Therefore it can be concluded that the use of solvent can be an option for formation of disulphide bridge as it is helping to dissolve the precursor completely and copper sulphate offers an added advantage towards increasing the rate of reaction.

Keywords: Peptide, Acetonitrile, Desmopressin Bis-SH, Ethanol, Methanol, copper sulphate.

1. INTRODUCTION

Oxidation of protein cysteine thiolates can be mediated by either specialized enzymes, such as protein disulfide

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isomerase (PDI), low molecular weight disulfide reagents, such as cystine, cystamine, oxidized glutathione or dithiothreitol, dithiobis(2-nitrobenzoic acid), or by other electron-accepting reagents such as oxygen (air), iodine, dimethyl sulfoxide, diamide and potassium ferricyanide.[4] Reaction can also be facilitated by also be done by copper chaperone.[27] The chemistry of disulphide bond formation is similar in proteins and smaller peptides. Whereas thiol/disulfide redox buffers are more applicable to larger polypeptides, cysteine-rich peptides can be oxidized by all types of organic and inorganic reagents. There are two important aspects of forming native disulfide bonds in proteins: (1) chemistry of thiol/sulfide exchange and (2) the kinetics and thermodynamics of oxidative folding. There are evidences that correctly folded species is a major oxidation product of the oxidation reaction. But it is mentioned in various reports that very poor folding yields for proteins and peptides. Reason for poor folding yields are not only from accumulation of misfolded species, but can be also occur due to accumulation of folding intermediates or kinetic traps. Since these by-products often have an increased non-polar surface area exposed to solvent, they show higher tendency for aggregation and precipitation.[4]

During formation of disulfide bonds in proteins or peptide intramolecular disulphide bond are favorable, but there are chances of formation of disulphide bond with another molecule i.e intermolecular disulphide bond formation. This competition between formation of the intramolecular bond and intermolecular disulfide bond dependant on concentration of the reaction, time required for reaction and medium of reaction. Since effective concentrations of cysteines in unfolded, reduced proteins typically range from 0.001 to 0.1 M, this imposes a requirement of using very dilute concentrations of proteins during the oxidative folding reaction.

Since use of cupric salts is reported to increase the rate of reaction therefore decided to use the same concept during cyclisation of desmopressin. The conventional way of disulphide bond formation is to dissolve the disulphide free species in water, adjust to basic pH and after complete consumption of free disulphide species stop the reaction by acidification. In the present research we have done modification such as use of mixture of water and solvents suitable for purification of peptides and use of catalytic amount of copper sulphate during cyclisation.

2. MATERIALS AND METHODS

Cyclisation of desmopressin Bis-SH was studied at concentration of 1mg/ml with and without catalyst with medium of reaction as Water, Water+$CuSO_4$, 50%ACN+50%WATER, 50%ACN+50%WATER+$CuSO_4$, 50%MEOH+50%WATER , 50%MEOH+50%WATER+ $CuSO_4$, 50% ETOH+50%WATER , 50% ETOH+50%WATER+ $CuSO_4$ as follows:

2.1 Method for reaction without copper sulphate

100 mg Desmopressin Bis-SH were weighed and dissolved separately in 100 ml of water, 50% ACN+WATER, 50% MEOH+WATER and 50% ETOH+WATER to perform reaction at 1 mg/ml concentration.

1. pH of the reaction was checked at the start and sample injected on HPLC as Before pH.
2. pH of the reaction was adjusted to 9.5±0.5 using ammonia solution
3. Progress of reaction was monitored by HPLC and pH was checked after regular interval.
4. After achieving the expected consume of the precursor (level below 1.5%) the reaction was stopped by addition of acetic acid.

2.2 Method for reaction with copper sulphate:

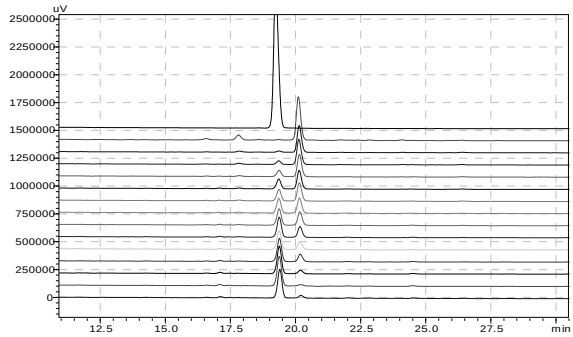
100 mg Desmopressin Bis-SH weighed and dissolved separately in 100 ml of water, 50% ACN+WATER, 50% MEOH+WATER and 50% ETOH+WATER to perform reaction at 1 mg/ml concentration.

1. pH of the reaction was checked at the start and sample injected on HPLC as Before pH.
2. pH of the reaction was adjusted to 9.5±0.5 using ammonia solution
3. 0.01 mg copper sulphate was added
4. Progress of reaction monitored by HPLC and pH was checked after regular interval.
5. After achieving the expected consume of the precursor (level below 1.5%) the reaction was stopped by addition of acetic acid.

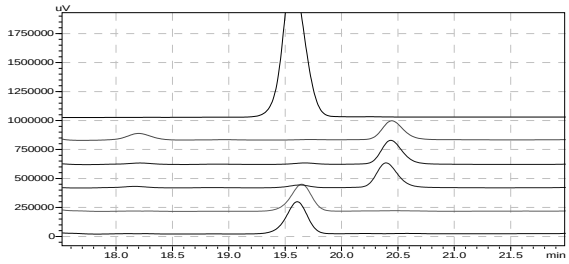
3. RESULTS AND DISCUSSION

3.1 Progress of reactions as follows

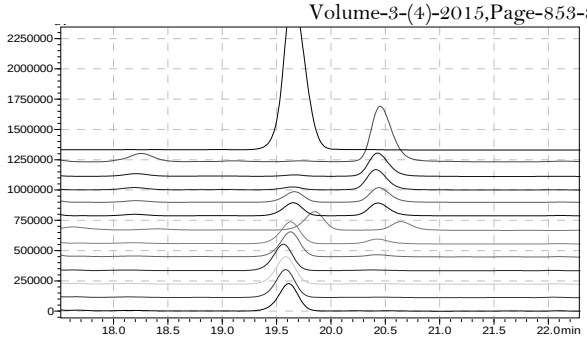
50%ACN+50%Water, 1 mg/ml concentration



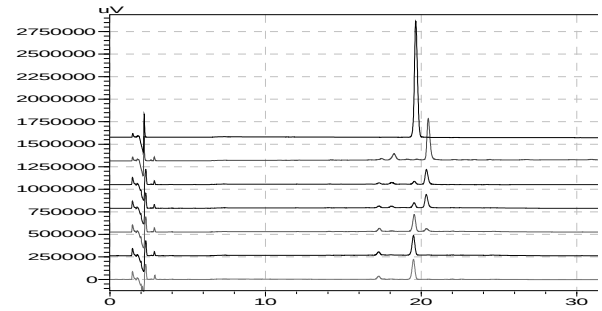
50%ACN+50%Water, 1 mg/ml concentration+$CuSO_4$



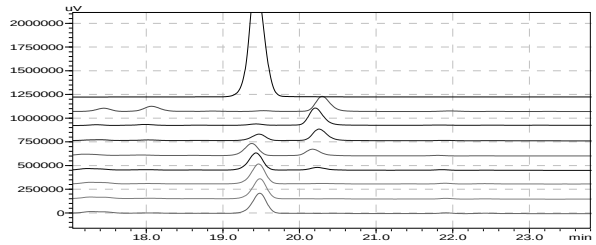
50%Ethanol+50%Water, 1 mg/ml concentration



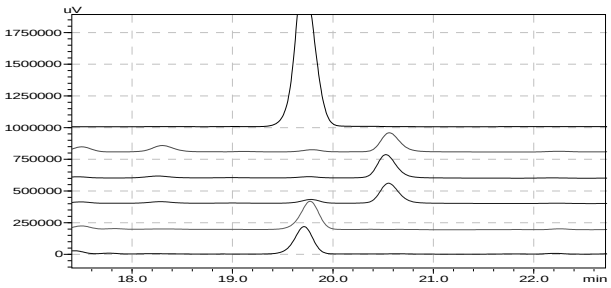
50% Ethanol +50%Water, 1 mg/ml concentration+$CuSO_4$



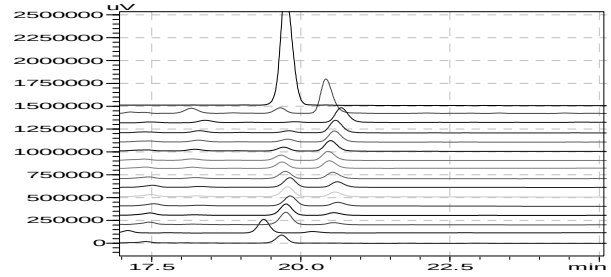
50% Methanol +50%Water, 1 mg/ml concentration



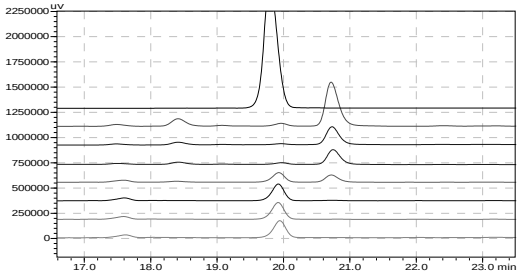
50% Methanol +50%Water, 1 mg/ml concentration+$CuSO_4$



Water, 1 mg/ml concentration



Water, 1 mg/ml concentration+$CuSO_4$



The overlapped chromatograms above are showing the progress of the oxidation in each reaction condition. The overlap is followed in the sequence from top to bottom as desmopressin std, desmopressin Bis-SH standard, reaction at 0 hours to completion of reaction. The first graph in each is overlap is of desmopressin std, second is of desmopressin bis-SH std, third is of 0 hours and then subsequent hours, the last graph is after acetic acid addition.

3.2 Details of Analytical Method used for Monitoring the Reaction:

1. Buffer A: Dissolve 9.08 gm of Potassium dihydrogen phosphate in 1 L water and add 5 ml of Trifluoroacetic acid and 5 ml Triethyl amine mix well and adjust pH to 5.01 with 10N NaOH.
2. Buffer B: 500 ml Buffer A+425 ml Acetonitrile+75ml Tetrahydrofuran.
3. Preparation of Desmopressin Standard Solution: Desmopressin Working Standard Vial dissolved in 25 ml of Milli-Q water.
4. Preparation of Desmopressin Bis-SH Standard Solution: 12.5 mg of desmopressin Bis-SH were dissolved in 25 ml of water.
5. Resolution Solution: Oxytocin Desmopressin validation mixture dissolved in water to obtain a concentration of 0.25mg/ml
6. Test Solution: 1 ml of sample diluted to 10 ml Milli-Q water.

Table 1: Column Specification

Column	C18, 250X4.6mm, 5 µ
Flow rate	1.5ml/min
Injection volume	50 µL for Blank, Std solution (Desmopressin and Desmopressin Bis-SH) 50 µL for Test solution
Run time	50 min

Table 2: Retention Time of Principal Peak

Sample Solution	**Retention Time of Principal Peak**
Desmopressin Std Solution	Around 19 min
Desmopressin Bis-SH Std Solution	Around 20 min
Resolution between main peak due to Desmopressin and main peak due to Oxytocin	NLT 1.5

Table 3: The results of experiments are tabulated in terms of Yield (Decreasing order) in following table.

Expt No.	Medium of reaction	concentration of reaction in mg/ml	Time required for completion in Hours	% yield as compared to area of desmopressin Bis-SH at start and Desmopressin at end of reaction	Purity of desmopressin
4	50%Acetonitrile+50%Water+CuSO4	1	3	156.63%	77.87%
19	Water+CuSO4	1	4	142.50%	71.30%
13	50%ETOH + 50%Water	1	17	124.26%	73.23%
10	50%MEOH + 50%Water+CuSO4	1	4	121.88%	75.13%
7	50%MEOH + 50%Water	1	10	114.74%	77.68%
22	Water	1	33	113.30%	56.06%
16	50%ETOH + 50%Water+CuSO4	1	5	109.96%	72.13%
1	50%Acetonitrile+50%Water	1	11	106.07%	74.76%

Table 4: The results of experiments are tabulated in terms of Purity (Decreasing order) required in following table.

Expt No.	Medium of reaction	concentration of reaction in mg/ml	Time required for completion in Hours	% yield as compared to area of desmopressin Bis-SH at start and Desmopressin at end of reaction	Purity of desmopressin
4	50%Acetonitrile+50%Water+CuSO4	1	3	156.63%	77.87%
7	50%MEOH + 50%Water	1	10	114.74%	77.68%
10	50%MEOH + 50%Water+CuSO4	1	4	121.88%	75.13%
1	50%Acetonitrile+50%Water	1	11	106.07%	74.76%
13	50%ETOH + 50%Water	1	17	124.26%	73.23%
16	50%ETOH +	1	5	109.96%	72.13%

	50%Water+CuSO4			
19	Water+CuSO4	1 4	142.50%	71.30%
22	Water	1 33	113.30%	56.06%

3.3 Cyclisation reaction in water:

In terms of purity: The cyclized desmopressin obtained by aerial oxidation at concentration 1 mg/ml, is having a purity of 56.06% and cyclized desmopressin obtained by aerial oxidation at concentration 1 mg/ml with copper sulphate is having purity of 71.30 % . Increase in purity indicates that the addition of copper sulphate accelerates disulphide scrambling leading the intramolecular reaction towards equilibrium.

***In terms of Time*:** The cyclisation reaction of desmopressin by aerial oxidation at concentration 1 mg/ml takes 33 hours and the cyclisation reaction of desmopressin by aerial oxidation at concentration 1 mg/ml with copper sulphate takes only 4 hours. The reaction using copper sulphate completed in 4 hours and indicates that the catalyst increases the speed of the reaction.

***In terms of Yield*:** Yield of the cyclisation reaction of desmopressin by aerial oxidation, at concentration of 1 mg/ml is 113.30% and cyclisation reaction of desmopressin by aerial oxidation at concentration 1 mg/ml with copper sulphate has a yield of 142.50%.

From above observations it is clear that the use of copper sulphate improves the reactions outcome.

3.4 Cyclisation reaction in Water: Acetonitrile:

***In terms of purity*:** The cyclized desmopressin obtained using Water: Acetonitrile as a solvent at concentration 1 mg/ml was having purity of 74.76% and cyclized desmopressin obtained using Water: Acetonitrile as a solvent at concentration 1 mg/ml with copper sulphate is having purity of 77.87 %. Increase in purity indicates that apart from addition of copper sulphate, use of solvent also improves the quality of end product by facilitating the intramolecular reaction to yield comparably higher purity product as compared to other experiments done in different solvents.

In terms of Time: The cyclisation reaction of desmopressin using Water: Acetonitrile as a solvent at concentration 1 mg/ml take 11 hours and cyclisation reaction of desmopressin using Water: Acetonitrile at concentration 1 mg/ml with copper sulphate takes only 3 hours. Reaction using copper sulphate complete in 3 hours, even reaction time with respect to reaction in Water: Methanol, Water: Ethanol was comparable, in terms of purity and yield it is better than any other experimental conditions tried during this experiments.

***In terms of Yield*:** Yield of **the** cyclisation reaction of desmopressin using Water: Acetonitrile as solvent at concentration of 1 mg/ml was 106.07% and cyclisation reaction of desmopressin using Water: Acetonitrile as solvent at concentration 1 mg/ml with copper sulphate had yield of 156.63%.

Considering overall Yield, time taken for completion of reaction and purity then the Water:Acetonitrile reaction using copper sulphate is preferable for cyclisation of peptides than Water:Methanol or Water:Ethanol solvent conditions

3.5 Cyclisation reaction in Water: Methanol:

In terms of purity The cyclized desmopressin obtained using Water: Methanol as solvent at concentration 1 mg/ml, was having purity of 77.68% and cyclized desmopressin obtained using Water: Methanol as solvent at concentration 1 mg/ml with copper sulphate was having purity of 75.13 %.

***In terms of Time*:** The cyclisation reaction of desmopressin using Water: Methanol as solvent at concentration 1 mg/ml took 10 hours and cyclisation reaction of desmopressin using Water: Methanol as solvent at concentration 1 mg/ml with copper sulphate took 4 hours. Reduced reaction time due to copper sulphate is indicative that it helps to speed up the reaction.

In terms of Yield: Yield of **the** cyclisation reaction of desmopressin using Water: Methanol as solvent at concentration of 1 mg/ml was 114.74% and cyclisation reaction of desmopressin using Water: Methanol as solvent at concentration 1 mg/ml with copper sulphate had yield of 121.88%.

Since the product obtained using Methanol: Waster+ copper sulphate as a solvent was showing purity of 75.13% and reaction time was 4 hours, but the yield of the reaction was only 121.88%(Acetonitrile:Water+ copper sulphate had 156.63%)therefore can be preferred for oxidation of desmopressin.

***3.6 Cyclisation reaction in Water: Ethanol*:**

***In terms of purity*:** The cyclized desmopressin obtained using Water: Ethanol as a solvent at concentration 1 mg/ml, was having purity of 73.23% and cyclized desmopressin obtained using Water: Ethanol as a solvent at concentration 1 mg/ml with copper sulphate had purity of 72.13 %. Which was comparably lower than that of experiments done in Water, Water: Acetonitrile and Water: Methanol.

***In terms of Time*:** The cyclisation reaction of desmopressin using Water: Ethanol as a solvent at concentration 1 mg/ml took 17 hours and cyclisation reaction of desmopressin using Water: Ethanol as a solvent at concentration 1 mg/ml with copper sulphate took only 5 hours. Reaction using copper sulphate completed in 5 hours which is longer as compared to time of reaction with the following reaction conditions: Water: acetonitrile + copper sulphate and Water: Methanol+ copper sulphate.

In terms of Yield: Yield of the cyclisation reaction of desmopressin using Water: Ethanol as a solvent at concentration of 1 mg/ml was 124.26% and cyclisation reaction of desmopressin using Water: Ethanol as a solvent at concentration 1 mg/ml with copper sulphate had yield of 109.96%.

4. CONCLUSION

From above observation it is clear that reaction using combination of acetonitrile, water and copper sulphate proved superior to all the other ones tested during cyclisation of desmopressin.

5. ACKNOWLEDGEMENT

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