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Papain-Catalyzed Synthesis of Oligolysine in Low-Water Organic Reaction Media

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ABSTRACT: Oligopeptides of L-lysine have the potential for applications in various scientific and technical areas. The number of residues in polycationic compounds such as oligolysine is also reported to have an effect on its biological properties. Hence, there is a necessity for developing efficient oligolysine synthesis methods where the oligopeptide dispersity can be tailored, along with optimum yield values. The ability of proteases to reverse their proteolytic activity to synthesize peptides has been reported in the literature. However protease-catalyzed synthesis of oligopeptides of basic amino acids such as lysine in aqueous buffers is hindered by unfavorable thermodynamics. In this work, a low-water organic system comprising an aqueous phase in contact with a bulk immiscible toluene solvent system has been demonstrated for efficient papain-catalyzed synthesis of oligolysine. The oligolysine mixture was separated with ion-pair LC. The LC peaks were identified with semipreparatory LC separation followed by solid-phase extraction purification and LC-MS analysis. The Plackett−Burman design method used to screen for significant variables showed that the oligolysine yield is strongly dependent on five variables, namely, the substrate concentration, the aqueous phase composition, the compositions of the additives 2-mercaptoethanol and *N*,*N*-diisopropylethylamine, and the duration of incubation. Using the one-variable-at-a-time approach, the effect of system variables on oligolysine dispersity was investigated. The results show that the dispersity profile can be tailored by modifying the magnitudes of these variables. The effect of these variables against oligolysine yield was investigated with response surface methodology. The results show that these variables interact with each other to produce a maximum yield of ∼92% with dispersity ranging from 2−10 lysine residues.

KEYWORDS: *dispersity, lysine, oligolysine, oligomers, oligopeptides, Plackett−Burman design, RSM*

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1. INTRODUCTION

Oligopeptides are known to play key roles in biological systems and are therefore considered as important targets for biochemical, pharmaceutical, nutritional, and cosmetic applications.[1,2] Oligolysine in particular has attracted attention with applications as (i) an essential amino acid lysine supplement for animal feed,[3,4] (ii) a coating agent for DNA for providing better protection against digestion by DNase I;[1] (iii) a medium for synthetic gene delivery, enhancing calcium absorption and maintaining bone health by preventing osteoporosis;[5,6] (iv) a key factor for cleaning arteries and cancer prevention;[7] (v) an oral medicine for preventing cold sores and genital herpes;[7] (vi) a tool for reducing anxiety;[7] and (viii) a wound healing agent.[8] It is well-known that polycationic compounds such as polylysine have antimicrobial properties.[9] However, there are few reports on whether the antimicrobial property depends on the number of charges on these compounds. Interestingly, pentalysine has been reported to bind with and increase the outer-membrane permeability of Gram-negative *Pseudomonas aeruginosa*.[10] In contrast to this, the trilysine and tetralysine peptides were found to be inactive, suggesting that the degree of polymerization is a decisive factor in the antimicrobial activity. Also, in a mixture of oligolysines, the degree of polymerization can be a crucial factor dictating the biological activity. To test this further, efficient methods for the synthesis and isolation of lysine oligopeptides with various degrees of polymerization are needed.

Oligopeptides may be synthesized with either chemical (solid-phase polypeptide synthesis, SPPS), microbial (recombinant technology), or enzymatic methods.[4] Chemical methods such as SPPS provide good control over peptide sequence and dispersity but suffer from relatively poor yields and the requirement of toxic solvents and cumbersome and convoluted amino acid protection and deprotection steps.[11] Enzymatic methods are more robust with high degree of purity, yield, stereospecificity, and regiospecificity, relatively mild reaction conditions, and low cost but suffer from wide peptide residue dispersity and identification of enzymes capable of good proteosynthetic as opposed to their natural proteolytic activity.[4,6,7,9−12] Enzymatic peptide synthesis maybe either (i) a thermodynamically controlled process, where the enzyme catalyzes the attainment of equilibrium leading to formation of peptides from monomers, or (ii) a kinetically controlled process, where the enzyme initially forms an acyl−enzyme

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intermediate with activated amino acid acyl donors in the form of esters or amides. The intermediate undergoes a nucleophilic attack by another amino acid to form a peptide bond.[13] In this case, the product peptide needs to be protected from secondary hydrolysis of the peptide bond[12−14]

The protease-catalyzed synthesis of hydrophobic amino acid oligopeptides in aqueous buffers from amino acid esters utilizing a combination of thermodynamically and kinetically controlled methods has been reported in the literature.[15,16] The product oligopeptides were reported to be protected from secondary hydrolysis by the precipitation of the increasingly hydrophobic growing chain of peptides. In contrast to this, the highly water-soluble basic amino acid oligopeptides do not precipitate and are prone to secondary hydrolysis, leading to poor yields.[16] A few reports have demonstrated the ability of low-water organic media possessing low water activity to support protease-catalyzed synthesis of basic amino acid oligopeptides.[17,18] The organic solvents can be introduced in these systems to form either water-miscible homogeneous or water-immiscible heterogeneous systems. The systems utilized a combination of thermodynamically and kinetically controlled synthesis to demonstrate protease-catalyzed oligopeptides synthesis with amino acid esters and amides.[19] However, such enzymatic synthesis of homo-oligopeptides of hydrophilic amino acids has been examined to a limited degree, and the effect of the various parameters on the dispersity and yield of the oligopeptides has not been examined. The cysteine protease papain, with its wide P1 site specificity, has been widely reported for proteosynthesis of oligopeptides.[20,21]

Herein we report the results of our investigation into the ability of the proteolytic enzyme papain to catalyze the synthesis of oligolysine in low-water toluene media. The system variables, namely, the compositions of the additives 2-mercaptoethanol and *N*,*N*-diisopropylethylamine (DIPEA), the aqueous (to organic) phase composition, the concentration of the substrate (L-lysine ethyl ester, L-Lys-OEt), the duration of incubation, the incubation temperature, and the stirring rate, were optimized in these systems for (i) determination of the yield and dispersity of the oligopeptide product with classical one-variable-at-a-time (OVAT) methodology and (ii) maximizing the oligopeptide yield with response surface methodology (RSM). The oligopeptide yield and dispersity were determined with ion-pair LC analysis. The identities of the individual peaks were established by collecting the individual peaks separated with semipreparatory ion-pair LC analysis, followed by solid-phase extraction (SPE) and LC-MS analysis. The OVAT results show that a maximum yield of ∼85% oligolysine can be obtained with the peptide dispersity ranging from 2 to 10 residues. The range and dispersity (degree of polymerization) of the lysine residues in the product could also be tailored by varying the parameters. The central composite design (CCD) results show that the substrate concentration, aqueous phase composition, additive compositions, and duration of incubation mutually interact with each other and play a significant role in determining the oligolysine yield, dispersity, and degree of polymerization. A maximum yield of ∼93% was obtained with the dispersity ranging from 2 to 10 residues in the oligolysine product.

2. MATERIALS AND METHODS

2.1. Materials. L-Lys-OEt·2HCl (purity ≥ 99%), papain from papaya latex (buffered aqueous suspension, 2× crystallized, ∼28 mg protein/mL, ∼19 units/mg of protein), 1-hexanesulfonic acid sodium salt (HSA) (purity ≥ 98%), 2-mercaptoethanol (purity ≥ 99%), DIPEA (purity ≥ 99%), α-cyano-4-hydroxycinnamic acid (CHCA) (ultrapure), 1,1,1,3,3,3-hexafluoro-2-propanol-d_2 (HFIP) (purity ≥ 99%), dilysine (Lys-Lys) (purity ≥ 99%), trilysine (Lys-Lys-Lys) (purity ≥ 97%), tetralysine (Lys-Lys-Lys-Lys) (purity ≥ 95%), and heptafluorobutyric acid (HFBA) (purity ≥ 98%) were purchased from Sigma-Aldrich Chemicals Pvt. Ltd., India. Water (HPLC grade), toluene (purity ≥ 99%), acetonitrile (HPLC grade, purity ≥ 99.9%), methanol (HPLC grade; purity ≥ 99%), orthophosphoric acid (purity ≥ 85%), ammonium hydroxide (purity ≥ 98%), and trifluoroacetic acid (TFA) (purity ≥ 99%) were purchased from Sisco Research Laboratories Pvt. Ltd. (SRL), India. Bond Elut Plexa PCX SPE cartridges (30 mg; 3 mL) were purchased from Agilent Technologies, USA. A Zorbax Eclipse XDB C-18 HPLC column (2.1 mm × 150 mm; 5 μm) supplied by Agilent Technologies was used for HPLC analysis. A Pursuit XRs C-18 HPLC semipreparatory column (250 mm × 10 mm; 10 μm) supplied by Agilent Technologies was used for the semipreparatory ion-pair LC separation. The software Minitab 14 (Minitab, LLC, USA), Design Expert 10 (StatEase, Inc., USA), and OriginPro 8.5.0 SR1 and Origin 2020 (Origin Lab Corporation, USA) were used in the investigation.

2.2. General Procedure for Protease-Catalyzed Synthesis of Oligolysine in the Two-Phase System. In the general synthesis procedure, 123 mg of L-Lys-OEt·2HCl (500 mmol, 50 mM) was weighed in a 22 mL glass vial with Teflon (PTFE/silicone septa)-lined screw caps. The 9.5 mL organic phase (95% v/v) consisting of 20 μL of 2-mercaptoethanol (0.2% v/v), 80 μL of DIPEA (0.8% v/v), and 9.4 mL of toluene (94% v/v) was added to the vial. The 0.5 mL aqueous phase (5% v/v) was formulated by adding ∼100−120 μL (∼3 mg) of papain solution made up with water to the vial. The final reaction mixture had an enzyme/substrate (E/S) ratio of 3.46% w/w. The 10 mL of low-water organic medium was stirred at 1000 rpm. At the end of 24 h incubation, the reaction mixture was heated to 90 °C to denature the papain, cooled, transferred to a 100 mL round-bottom flask, and dried in a rotary evaporator at 45 °C to recover the product residue. The dry contents were resuspended in water and further diluted for ion-pair LC analysis. All experiments were performed in triplicate.

2.3. Sample Preparation and Analysis. *2.3.1. Ion-Pair LC Separation.* An Agilent 1220 Infinity HPLC system coupled with a variable-wavelength UV detector (VWD) was used for the analysis. The mobile phase consisted of eluent A (10 mM HSA and 0.1% H_3PO_4 in water) and eluent B (10 mM HSA and 0.1% H_3PO_4 in 50% acetonitrile in water solution). The oligolysine synthesis samples were separated with ion-pair LC on a Zorbax Eclipse XDB C-18 column (2.1 mm × 150 mm; 5 μm) supplied by Agilent Technologies. The separation was carried out by varying the solvent gradient from 80% A to 100% B in 30 min at a flow rate of 0.2 mL/min and 40 °C followed by detection at 210 nm with the VWD.

2.3.2. Semipreparatory Ion-Pair LC Separation. The Agilent 1220 Infinity HPLC system coupled with a VWD was used for the semipreparatory LC separation. The mobile phase consisted of eluent A (10 mM HSA and 0.1% H_3PO_4 in water) and eluent B (10 mM HSA and 0.1% H_3PO_4 in 50% acetonitrile in water solution). The oligolysine synthesis samples were separated with ion-pair LC on a Pursuit XRs C-18 column (250 mm × 10.0 mm; 10 μm) supplied by

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Agilent Technologies. The separation was carried out by varying the solvent gradient from 80% A to 20% B in 30 min at a flow rate of 5 mL/min and 40 °C followed by detection at 210 nm with the VWD. The individual bands eluting from the HPLC were collected in sample tubes, dried in the rotary evaporator, and resuspended in 5 mL of water for further processing.

2.3.3. Solid-Phase Extraction. The bands collected in the ion-pair semipreparatory LC were purified with SPE to isolate the oligolysine sample from the ion-pairing agent HSA used in the chromatography. In the SPE method, the strong cation exchange Bond Elut Plexa PCX SPE cartridge was pretreated with 5 mL of 2% orthophosphoric acid solution, conditioned with 10 mL of methanol, and equilibrated with 10 mL of water before loading of the sample. The cartridge was washed with 5 mL of a 2% acetic acid solution. In the first elution step, 5 mL of 50% methanol in an acetonitrile solution was used to elute acids and neutral impurities. In the second elution step, 5 mL of 5% ammonium hydroxide in 50% methanol in acetonitrile solution was used to elute the basic oligolysine sample. This eluent solution was dried in the rotary evaporator, and the contents were resuspended in water for further LC-MS analysis.

2.3.4. Liquid Chromatography–Mass Spectrometric Analysis. The LC-MS system (Model LCMS 2020, Shimadzu, Japan) consisted of a Shimadzu HPLC mated to a single-quadrupole MS detector. The LC utilized a linear gradient program consisting of mobile phase A (0.1% HFBA in water) and mobile phase B (0.1% HFBA in 50% acetonitrile in water solution). The LC separation was carried out at a flow rate of 0.2 mL/min with solvent composition from 75% A to 50% B in 30 min. The chromatograms were studied at a wavelength of 210 nm using a UV detector. The MS analysis was carried out in full scan mode from m/z 200 to 1000.

2.4. Plackett–Burman Design to Determine Predominant Variables. In the first step, the statistical Plackett–Burman (PB) design method was employed using Minitab 14 software to screen and determine the predominant variables that are likely to affect the yield of the oligolysine product. In contrast to a full-factorial experiment, the PB model utilizes a minimum number of experiments to screen for significant variables when it is possible to neglect higher-order interactions and utilize two-level multifactor experiments. However, it does not provide any information about the dependence of one variable on another. The seven variables and the codes used to denote them in the software were aqueous phase composition (A), stirring rate (B), incubation temperature (C), 2-mercaptoethanol (D), DIPEA (E), duration of incubation (F), and substrate concentration (G). These variables can be fed into the software with "+1" and "−1" codes for high and low levels of the variables, respectively. The PB design suggests a minimum number of experimental runs that can be conducted and entered as the response variable in the software. In this case, the PB design suggested 12 experimental runs as shown in Table 1.

2.5. One-Variable-at-a-Time Method for Yield and Dispersity Profile of the Oligolysine Product. The OVAT method is designed to study the response of variables one at a time while holding all other variables constant. In contrast to designs that study the effect of multiple variables simultaneously, OVAT requires more experimental runs and is not useful for estimating the interactive effects of the variables. In this second step, the effect of predominant variables on the yield and dispersity of lysine residues in the product was studied with the OVAT methodology. The effect of varying the aqueous phase composition (A), 2-mercaptoethanol (B), DIPEA (C), substrate concentration (D), and duration of incubation (E) were investigated. The yield, dispersity, and individual peak area counts were recorded from the ion-pair LC separation analysis described in section 2.3.1.

Table 1. Plackett–Burman Design Table for Determining the Predominant Variables Influencing the Synthesis of the Oligolysine Product[a]

run order	A (%)	B (rpm)	C (°C)	D (%)	E (%)	F (h)	G (mM)	yield (%)
1	10	200	80	0.02	0.08	0.5	300	63.86
2	10	1000	20	2.00	0.08	0.5	50	78.37
3	1	1000	80	0.02	8.00	0.5	50	58.77
4	10	200	80	2.00	0.08	24.0	50	52.55
5	10	1000	20	2.00	8.00	0.5	300	85.99
6	10	1000	80	0.02	8.00	24.0	50	57.93
7	1	1000	80	2.00	0.08	24.0	300	76.11
8	1	200	80	2.00	8.00	0.5	300	70.88
9	1	200	20	2.00	8.00	24.0	50	78.58
10	10	200	20	0.02	8.00	24.0	300	72.53
11	1	1000	20	0.02	0.08	24.0	300	80.86
12	1	200	20	0.02	0.08	0.5	50	81.31

[a]The statistical analysis of these results was used to generate a table and highlight variables with $p < 0.05$, which can be used to select variables that may be considered as sufficiently significant to influence the oligolysine product yield as described in section 3.5: (A) aqueous phase ratio; (B) stirring rate; (C) incubation temperature; (D) 2-mercaptoethanol; (E) DIPEA; (F) duration of incubation; (G) substrate concentration.

2.6. Central Composite Design to Maximize the Yield of Oligolysine Product. In the third step, a CCD-based statistical optimization method using the Design Expert 10 software was employed. This was used to study the interaction effects among the predominant variables indicated by the PB design experiments and determine the optimum magnitudes of the variables providing a maximum yield. In this method, five variables (minimum and maximum values in parentheses), namely, aqueous phase composition (1–10%), 2-mercaptoethanol (0.02–2% v/v), DIPEA (0.08–8% v/v), substrate (L-Lys-OEt) concentration (50–300 mM), and duration of incubation (0.5–24 h) were labeled as factors A, B, C, D, and E respectively. The experimental matrix with 50 runs suggested by the CCD model based on the number of variables to be optimized is produced in Table 3. The yield results from these runs were recorded, and statistical analyses, including Analysis of Variance (ANOVA) and multiple regression, were carried out to study the interactive effects among the variables and optimize the oligolysine product yield.

3. RESULTS AND DISCUSSION

3.1. Synthesis of Hydrophilic Amino Acid Oligopeptides. Peptide bond synthesis is a reversal of the thermodynamically favorable hydrolysis reaction. Proteases, with their ability to accelerate reactions at near-neutral-pH conditions, ambient temperature, and without any byproducts, have been the catalyst of choice for such reverse reactions, i.e., proteosynthesis as against the naturally assumed proteolysis action. Two types of strategies, (i) thermodynamically controlled and (ii) kinetically controlled, have been employed for such protease-catalyzed peptide bond synthesis reactions.

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The thermodynamically controlled strategy is a direct reversal of the hydrolysis reaction with free amino acid acyl donors and acceptors. It consists of two steps, an endergonic ionization−neutralization step of the zwitterionic-form amino acids followed by the exergonic peptide synthesis step between the nonionized amino acid acyl donor and acceptor amino acids. However, the overall process is endergonic with poor peptide yields at equilibrium, making it a poor choice for the synthesis.[22]

The kinetically controlled peptide synthesis utilizes activated acyl donors and is primarily a transamidation reaction of activated amino acids. The synthesis is possible only by cysteine and serine proteases with double addition−elimination steps shown in Figure 1. This figure is a schematic of

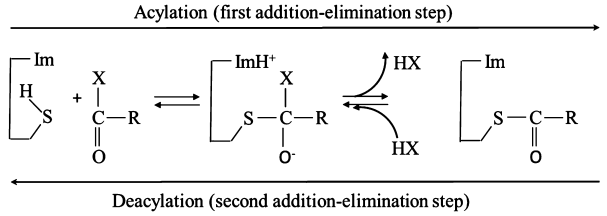

Figure 1. Schematic of peptide bond synthesis mechanism in cysteine proteases (Im = imidazole moiety of histidine residue in the peptide chain; −SH = active-site cysteine residue side-chain moiety; R = moiety attached to the α-carbon atom of the amino acid; X = leaving group moiety attached to the α-carbon atom of the amino acid).

the peptide bond synthesis mechanism in cysteine proteases, such as papain. In the first acylation step, the active-site nucleophilic cysteine residue (−SH) initially donates a proton to a histidine imidazole (Im) group in the active site. This facilitates a nucleophilic attack on the carbonyl carbon of the acyl donor (amino acid, R = moiety attached to the α-carbon atom of the amino acid, and X = leaving group moiety attached to the α-carbon atom of the amino acid) and the formation of an unstable tetrahedral intermediate. The intermediate decomposes to form an acyl−enzyme complex and a leaving group (HX). If the acyl group is a free amino acid, then HX is water. If the acyl group is an activated form, such as amino acid ester, then HX is an alcohol. In this step, the endergonic acyl−enzyme complex-forming step is coupled with the auxiliary exergonic ester hydrolysis step, making the overall reaction exergonic. In the second step, which is a reversal of the first step, the acyl−enzyme complex can undergo competitive deacylation through aminolysis or hydrolysis with either an amine or water, respectively. If HX is water, then the second step is a hydrolysis step producing the free amino acid product. If HX is an amino acid ester, then it produces a dipeptide product with a C-terminal ester. The dipeptidyl ester product can be the substrate for the second round of reaction to form an acyl−enzyme complex, which can form a tripeptidyl ester product, and so on, leading to the growth of the amino acid chain and formation of the oligopeptide product. The growth of the amino acid chain is terminated in the second step due to either the hydrolysis with water or reaction with a free amino acid to form a peptide with a C-terminal hydroxyl group bearing. However, the product oligopeptides are again susceptible to secondary hydrolysis catalyzed by the same enzyme in the first step. Hence, the product in such reactions is usually a mixture of peptides with different chain lengths. With sufficient duration of incubation, the product peptides can undergo extensive hydrolysis and produce a poor peptide yield at equilibrium. Hence, in kinetically controlled synthesis reactions, high yields can be realized only if the reaction is terminated before the equilibrium is attained.[23,24] The kinetically controlled strategy can be adopted for the protease-catalyzed oligopeptide synthesis to realize high yields if the product oligopeptide can be transported away from the reaction medium, such as by precipitation or partition into a water-immiscible nonpolar solvent, making it relatively inaccessible for the secondary hydrolysis.

Hydrophilic amino acids such as lysine have good solubility in aqueous media but suffer from limited solubility in nonpolar media. When the enzyme-catalyzed syntheses of these oligopeptides are conducted in a buffered aqueous medium, the products are highly soluble in the medium, making them susceptible to secondary enzymatic hydrolysis, thereby lowering the yield and dispersity of the oligopeptide product.[25,26] This may be solved by conducting the synthesis in either (i) a low-water-activity medium such as a monophasic water-miscible protic-solvent-based mixed medium (e.g., water/acetonitrile) or (ii) a low-water organic biphasic medium such as the low-water toluene system reported in this work. Available literature shows that the monophasic water/acetonitrile media provided yields as low as 35% with mainly dipeptide products, making them unattractive for oligopeptide synthesis.[27] This is most probably due to the reduced catalytic activity and stability of the enzyme in the medium. Low-water-content organic media, on the other hand, though reported for oligopeptide synthesis, have not been characterized for the effect of the variables on dispersity and maximizing the yield of the oligopeptide product.[28−32]

To obtain the desired yield and oligopeptide dispersity, it is crucial to select the right protease capable of catalyzing the reverse proteosynthesis reaction and the right reaction medium, organic solvents, and additives (if any) used to stabilize the enzyme activity. Papain, a cysteine protease, is capable of forming the acyl−enzyme complex. It has been reported for its wide specificity and ability to catalyze the proteosynthesis reactions in low-water media with yields as high as 80%. Hence, it was chosen as the enzyme for the kinetically controlled oligolysine synthesis in the low-water toluene reaction system.[33−37]

The low-water reaction medium should consist of an organic solvent that has poor mutual solubility with water but is capable of partitioning or extracting the growing peptide chain away from the water phase and hosting it in either the bulk or at the water−solvent interphase, thereby hindering the secondary hydrolysis of the peptide chain. Hence, the organic solvent needs to be chosen on the basis of the following desirable factors: (i) immiscibility with water to form the heterogeneous phase system and (ii) ability to partition the reactants, products, and byproducts appropriately to facilitate the shifting of the equilibrium toward synthesis. In the case of enzyme-catalyzed reactions, the organic solvent should also be relatively immiscible with water to prevent enzyme denaturation but be able to partition the relatively hydrophilic product away from the aqueous phase to enable the shifting of the equilibrium toward synthesis. Thus, a highly hydrophobic solvent may be able to form the low-water organic heterogeneous system but not be able to partition the product away. In contrast, a relatively hydrophilic solvent that is partly (or completely) water-miscible may not be able to retain enzyme activity. A judicious selection of the solvent may be

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done with the aid of the log *P* values of solvents. A solvent with a negative log *P* value is considered hydrophilic and miscible with water, making it a poor choice for enzyme-catalyzed reactions in low-water organic reaction media. In contrast to this, solvents with log *P* values >5 are considered very hydrophobic and might not be able to partition the relatively hydrophobic oligolysine product. Hence, a solvent with a moderate log *P* value with low vapor pressure is needed for this system. Toluene with log $P \approx 2.7$ and boiling point = 110.6 °C is one of the best choices for this low-water reaction medium.[38] In fact, toluene has already been reported as a good solvent for such reactions and was chosen for the reaction medium.[37,39,40] In low-water organic media, the additives 2-mercaptoethanol and DIPEA have been reported to be essential for oligopeptide synthesis. 2-Mercaptoethanol is a known reducing agent and is used for the stability of the disulfide bond in papain. The presence of at least 0.2% v/v 2-mercaptoethanol is reported to be essential for efficient oligopeptide synthesis. The presence of DIPEA is considered essential to neutralize the S1 subsite of papain when cationic substrates such as hydrophilic amino acids are used.[39] Hence, the additives 2-mercaptoethanol and DIPEA were utilized in the oligopeptide synthesis. This resulted in the formulation of low-water organic media for the oligolysine synthesis consisting of water, toluene, 2-mercaptoethanol, and DIPEA along with the protease (papain) and the substrate (L-Lys-OEt), as detailed in section 2.2.

3.2. ION-PAIR LIQUID CHROMATOGRAPHY ANALYSIS OF OLIGOLYSINE SAMPLE

Initially, the general procedure mentioned in section 2.2 was used to synthesize the oligolysine product in the low-water toluene medium. The constituents of the sample were evaporated to dryness in a rotary evaporator and resuspended in water. The solution was diluted with water, and a 10 μL aliquot of the sample was injected into the LC for ion-pair liquid chromatographic separation as described in section 2.3.1. The UV trace of the chromatogram obtained is shown in panel LC1 of Figure 2.

The chromatogram shows a series of peaks, which are likely to be oligomer homologues with varying numbers of lysine residues. The identity of the peaks eluting at 17.3 and 18.9 min were readily confirmed by matching with the retention times of the standard dilysine and trilysine samples, respectively, as shown in panels LC2 and LC3 of Figure 2. As standards were not available, the identities of the rest of the peaks were confirmed as discussed in the sections that follow.

3.3. Semipreparatory Ion-Pair Liquid Chromatography Analysis and Solid-Phase Extraction of Oligolysine Sample. To determine the identities of the peaks in panel LC1 of Figure 2 eluting at 20.5, 21.9, 22.6, and 23.1 min, etc. in the oligolysine sample, the sample was separated on a semipreparatory ion-pair liquid chromatography column with UV detection as described in section 2.3.2. The individual bands eluting from the column from various chromatographic separation runs were collected, pooled together, dried in a rotary evaporator, and resuspended in water. A 10 μL aliquot of each sample was injected into the ion-pair LC and analyzed as described in section 2.3.1. The chromatograms from four of these samples are shown in panels LC4, LC5, LC6, and LC7 of Figure 2. The chromatograms were found to have a single peak with a retention time of 20.5, 21.9, 22.6, and 23.1 min, respectively, matching with the elution times of four peaks in panel LC1 of Figure 2. The absence of any additional peaks in the chromatograms was also used to establish the purity of the peak isolates. The samples corresponding to retention times of 17.3, 18.9, 20.5, 21.9, 22.6, 23.1 min, etc. in Figure 2 were labeled as LC2, LC3, LC4, LC5, LC6, LC7, etc., respectively, for further analysis.

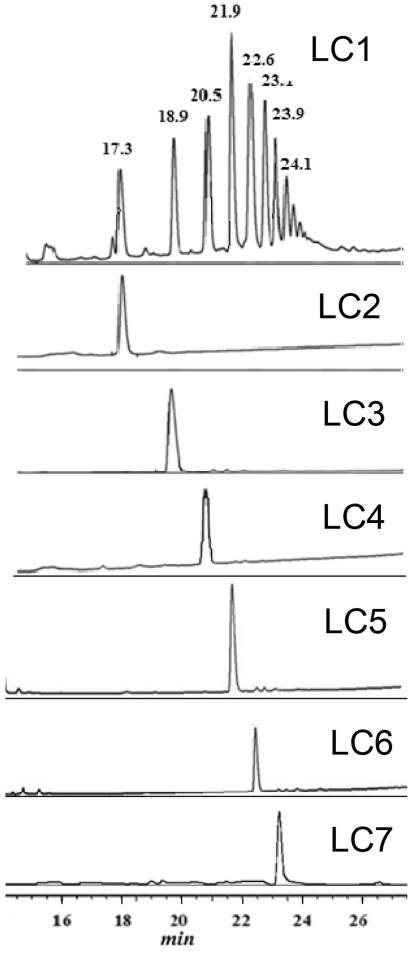

Figure 2. Ion-pair liquid chromatography separation of the sample analyzed as mentioned in section 2.3.1. LC1: oligolysine sample showing a series of peaks with retention times of 17.3, 18.9, 20.5, 21.9, 22.6, 23.1, 23.9, and 24.1 min. LC2: chromatogram of a standard dilysine sample showing a peak with retention time of 17.3 min, corresponding to the peak eluting at 17.3 min in LC1. LC3: chromatogram of a standard trilysine sample showing a peak with a retention time of 18.9 min, corresponding to the peak eluting at 18.9 min in LC1. LC4, LC5, LC6, and LC7: peaks identified as tetralysine, pentalysine, hexalysine, and heptalysine, corresponding to the sample peaks eluting at 20.5, 21.9, 22.6, and 23.1 min in LC1 after semipreparatory LC followed by SPE purification and LC-MS analysis as described in sections 2.3.2, 2.3.3, and 2.3.4 respectively.

To establish the identity of these samples, LC-MS analysis was employed. However, the samples contained abundant amounts of the nonvolatile ion-pairing agent HSA used in the semipreparatory ion-pair LC analysis. As the nonvolatile HSA was expected to interfere with LC-MS analysis, the oligolysine peak isolate samples were purified with SPE as described in section 2.3.3. The eluents from the SPE were freeze-dried, resuspended in water, and injected for ion-pair LC separation as described in section 2.3.1 to reconfirm the retention times and identities of the sample peaks as shown in Figure 2.

3.4. Liquid Chromatography−Mass Spectrometry Analysis of Oligolysine Samples. The identities of the

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oligopeptides peaks were established with LC-ESI$^+$-MS analysis as described in section 2.3.4. An aliquot of peak isolate sample labeled LC2 corresponding to the peak eluting at 17.3 min in panel LC1 of Figure 2 obtained after semipreparatory LC separation followed by SPE was injected into the LC-MS. Panel MS2 of Figure 3 depicts the base-peak

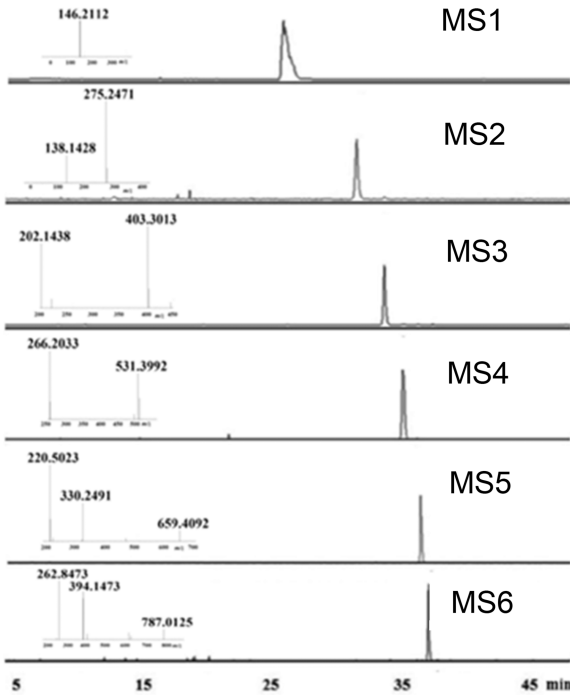

Figure 3. LC-ESI$^+$-MS analysis base-peak ion chromatograms of semipreparatory ion-pair LC-isolated and SPE-purified oligolysine sample peaks as described in sections 2.3.2, 2.3.3, and 2.3.4, respectively. The ion chromatograms were used to identify oligolysine homologues with singly, doubly, and triply protonated dilysine, trilysine, tetralysine, pentalysine, and hexalysine samples in panels LC2, LC3, LC4, LC5, and LC6 respectively of Figure 1.

ion chromatogram of the LC-MS analysis with this sample, showing the presence of singly and doubly protonated dilysine ($[M + H]^+ = m/z$ 275.2; $[M + 2H]^{2+} = m/z$ 138.1).

Similarly, aliquots of peak isolates labeled LC3 and LC4 corresponding to the peaks eluting at 18.9 and 20.5 min, respectively, in panel LC1 of Figure 2 when subjected to LC-MS analysis produced the base-peak ion chromatograms shown in panels MS3 and MS4 of Figure 3. The ion chromatograms show the presence of a base peak and an additional peak corresponding to the singly and doubly protonated trilysine ($[M + H]^+ = m/z$ 403.3; $[M + 2H]^{2+} = m/z$ 202.15) and tetralysine ($[M + H]^+ = m/z$ 531.4; $[M + 2H]^{2+} = m/z$ 266.2), respectively. The above results reconfirm the results of comparison with the retention time with standard dilysine and trilysine samples reported in section 3.2. The identity of sample LC5, corresponding to the peak eluting at 21.9 min in panel LC1 of Figure 2, was established as pentalysine with the ion chromatogram shown in panel MS5 of Figure 3, which shows the presence of three peaks at $[M + H]^+ = m/z$ 659.4, $[M + 2H]^{2+} = m/z$ 330.2, and $[M + 3H]^{3+} = m/z$ 220.5 corresponding to singly, doubly, and triply protonated pentalysine, respectively. Sample LC6 corresponding to the peak eluting at 22.6 min in panel LC1 of Figure 2 was established as hexalysine from the results shown in panel MS6 of Figure 3. The ion chromatogram was found to have peaks corresponding to singly, doubly, and triply protonated hexalysine ($[M + H]^+ = m/z$ 787.5, $[M + 2H]^{2+} = m/z$ 394.2, and $[M + 3H]^{3+} = m/z$ 263.1, respectively). The identities of the other peaks in panel LC1 of Figure 2 were all established similarly and found to be higher lysine oligopeptide homologues. These results put together establish that the low-water toluene system can effectively host the papain-catalyzed synthesis of lysine oligopeptides. The resultant peptide product appeared to have a dispersity ranging from 2 to 12 lysine residues. However, the effect of the process variables on the dispersity and oligolysine yield was yet to be investigated.

3.5. Plackett–Burman Design Study to Determine Predominant Variables Affecting Oligolysine Synthesis.

The papain-catalyzed synthesis of lysine oligopeptides in the low-water toluene system has been demonstrated; however, the effect of variables on the oligopeptide yield and dispersity needs to be investigated. Statistical methods based on the Plackett–Burman design with the Minitab software were used to identify the variables impacting the yield of oligolysine as described in section 2.4. This was also used to select the predominant variables and study their interactive effects with CCD as discussed in section 2.6. The seven variables and (in parentheses) the codes used to denote them in the software and the suggested range of values were the aqueous phase composition (A; 1–10%), stirring rate (B; 200–1000 rpm), incubation temperature (C; 20–80 °C), 2-mercaptoethanol (D; 0.02–2% v/v), DIPEA (E; 0.08–8% v/v), duration of incubation (F; 0.5–24 h), and substrate (L-Lys-OEt) concentration (G; 50–300 mM). These variables were fed into the software as "+1" for high level and "−1" for low level. The 12 experimental runs with details suggested by the design as listed in Table 1 were conducted, and the oligolysine product yields were recorded as the response in the last column of Table 1 of the software. The tabulation shows that the yield ranged from 85.99% for trial run number 5 to a low of 52.55% for trial run number 4. A regression analysis of the response data utilizing a confidence level of 95%, $\alpha = 0.05$, and $R^2 = 93.39$ was used to obtain Table 2.

Table 2. Results of the Statistical Optimization with the Plackett–Burman Design to Determine the Predominant Variables Influencing Oligolysine Synthesis (A: Aqueous Phase Ratio; B: Stirring Rate; C: Incubation Temperature; D: 2-Mercaptoethanol; E: DIPEA; F: Duration of Incubation; G: Substrate Concentration).

term	effect	coefficient	SE coefficient	t	p
A (%)	−6.197	−3.098	1.459	−2.21	0.017
F (h)	−2.703	−1.352	1.459	−5.45	0.041
E (%)	−15.907	−7.953	1.459	−5.45	0.022
D (%)	4.22	2.11	1.459	1.45	0.006
B (rpm)	−1.08	−0.54	1.459	−0.37	0.73
C (°C)	−3.087	−1.543	1.459	−1.06	0.35
G (mM)	7.437	3.718	1.459	2.55	0.033

The statistical analysis of the results of Table 1 was used to generate Table 2 and highlight variables with $p < 0.05$, which were considered as sufficiently significant to influence the oligolysine product yield. The five predominant variables which were determined by the PB design analysis to influence the yield were the aqueous phase composition (A), 2-mercaptoethanol (D), DIPEA (E), duration of incubation

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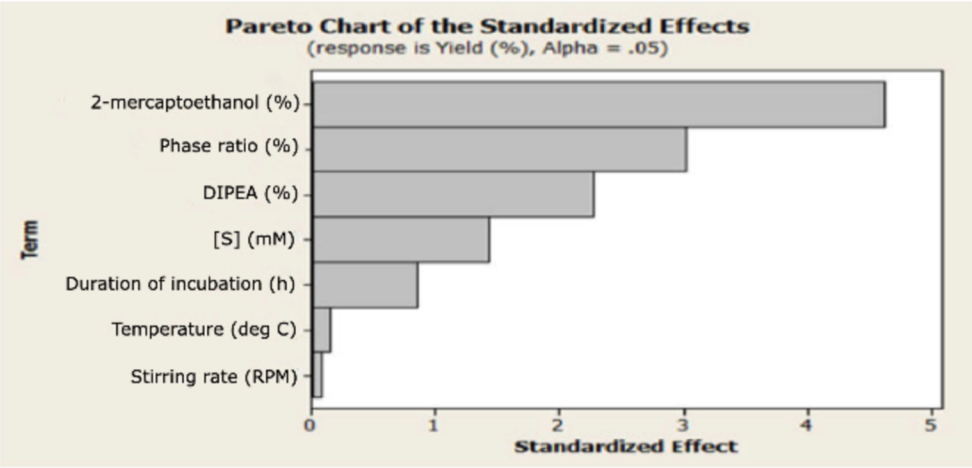

Figure 4. Pareto chart obtained from the PB design analysis listing all the predominant factors that affect the yield of oligolysine.

(F), and substrate concentration (G). The p values also suggest that the stirring rate (B) and incubation temperature (C) were not significant enough to affect the yield. This was also confirmed with the Pareto chart in Figure 4. The chart visually depicts the significance of the large number of variables affecting a process in the form a bar graph. The bars are ranked in hierarchical order with most significant variable at the top, as shown in Figure 4. Utilizing a confidence level cutoff of 95%, the first five ranked variables were found to be 2-mercaptoethanol (D), aqueous phase composition (A), DIPEA (E), substrate concentration (G), and duration of incubation (F). These variables were designated as the predominant variables and used with the OVAT and statistical CCD optimization studies as described in the following sections.

3.6. One-Variable-at-a-Time Study of the Effect of Predominant Variables on the Yield and Dispersity of Oligolysine Product. OVAT is a classical design strategy where the responses of individual variables are studied one at a time while keeping the other variables constant. The OVAT method compared to the more economical factorial design methods cannot study the effect of multiple variables simultaneously. It is also not able to estimate the magnitude of interactive effects among the variables studied. OVAT also requires many experimental runs but in general is preferred when the experimental runs are inexpensive, not time-consuming, and cumbersome. In this investigation, the variables can affect the overall yield as well as the range of dispersity of the oligolysine product mixture. Statistical methods such as CCD, which optimizes for one dependent variable (say, the yield), cannot be utilized to study the dispersity. Hence, in this step, the effect of predominant variables on the yield and dispersity of lysine residues in the product was studied with the OVAT methodology. The effects of varying the aqueous phase composition (1−10% aqueous in organic); 2-mercpatoethanol (0.02−2% v/v), DIPEA (0.08−8% v/v); substrate (L-Lys-OEt) concentration (50−300 mM), duration of incubation (0.5−24 h), and incubation temperature (20−80 °C) were investigated. The oligolysine yield was obtained based on the residual lysine monomer detected with the ion-pair LC analysis described in section 3.2. The area counts of the individual peaks in the LC traces were recorded and used as a measure of the abundance of the individual homologues in the oligolysine product sample.[41]

3.6.1. Effect of Aqueous Phase Composition. In enzyme catalysis in aqueous media, when the products are retained in solution, they are susceptible to the reverse reaction, hindering high conversions. This can be prevented by transporting the product away from the aqueous medium, making it inaccessible for further enzymatic reactions. In protease-catalyzed peptide synthesis with hydrophobic amino acids, this has been effectively achieved by salting-out and precipitating the growing hydrophobic peptide chains in high-ionic-strength media. Hydrophilic amino acid peptides with good solubility in water are not amenable to this strategy, making them susceptible to the secondary hydrolysis reaction leading to poor yield and dispersity. This problem can be solved by conducting the reaction in a low-water-content nonpolar medium, which reduces the water activity and also extracts the growing peptide chain away from the aqueous medium into the bulk nonpolar phase. Hence, the ratio of the aqueous phase to the toluene phase is expected to play a crucial role in determining the dispersity and yield of the oligolysine. This was investigated by maintaining all the variables as listed in section 2.2, except for the water content, which was varied from 0 to 10% v/v. The product sample was processed and subjected to ion-pair LC analysis, as described in section 2.3.1. The peaks in the chromatogram were assigned based on the results of sections 3.2, 3.3, and 3.4. This was used to calculate the yield based on the residual lysine monomer amount in the sample. The area counts of the oligolysine peaks in the chromatogram were tabulated and plotted as shown in Figure 5a.

The plot shows that the dispersity and yield of oligolysine vary considerably when the water content is varied from 0 to 10% v/v. When the reaction was conducted in neat toluene in the absence of any water content, no oligolysine yield was observed. This is most probably due to inactivation of the papain in the organic solvent without any water hydration necessary to maintain activity. When the water content was raised and varied from 1 to 1.67%, the oligolysine yield was observed to be constant at ∼85% with very narrow dispersity consisting of mainly trilysine and tetralysine and the poor presence of higher-order oligopeptides. When the water content was increased beyond this to 2.5 and 5%, the yield was still maintained at ∼85%, but higher-order oligolysines were found to be formed with wider dispersity ranging from two to eight residues. It was also found that in these samples,

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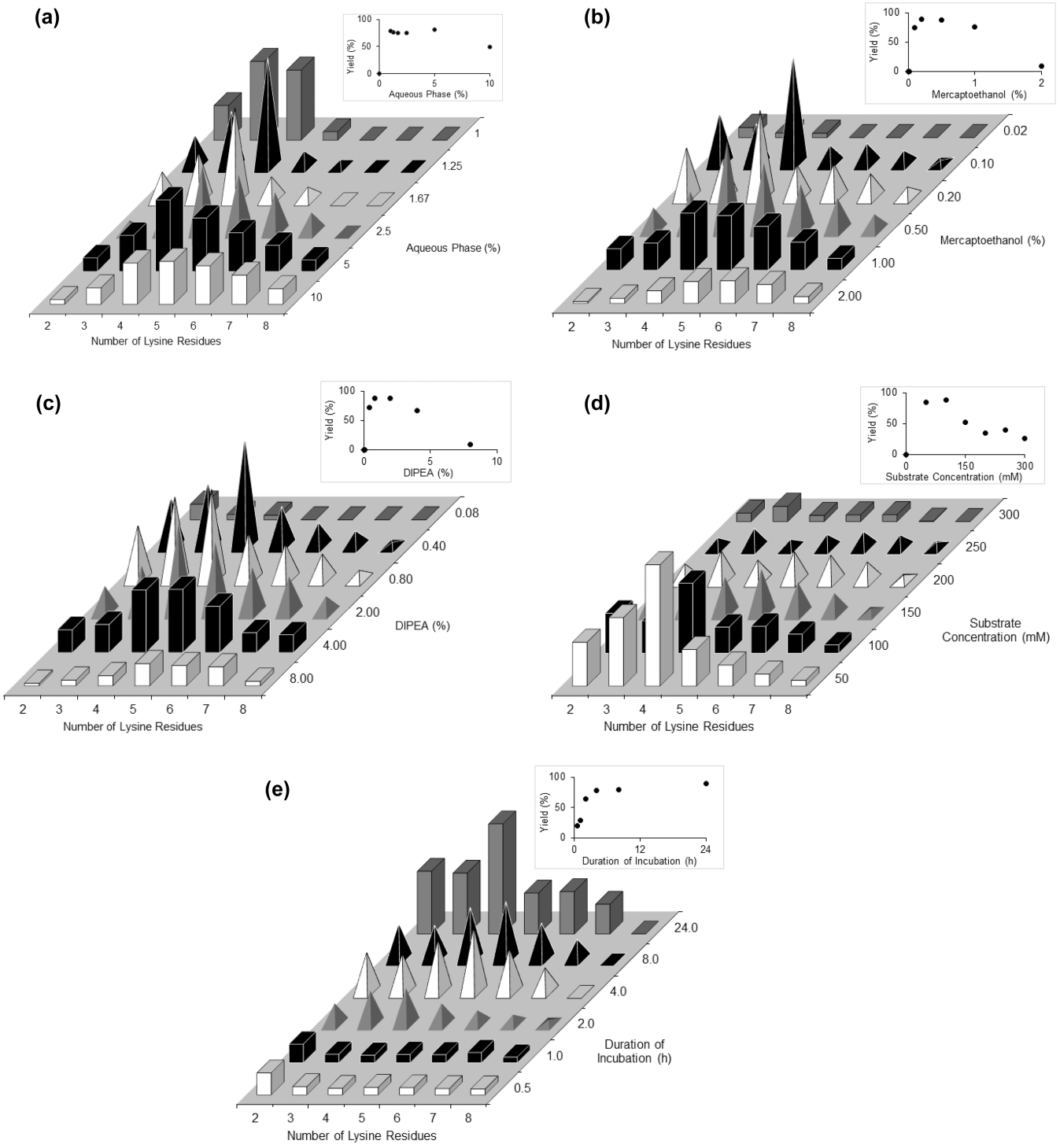

Figure 5. (a) Effect of aqueous phase composition (100−1000 μL, 1−10% v/v) on the yield and dispersity of oligolysine in low-water toluene media (L-Lys-OEt: 0.5 mmol, 50 mM; 2-mercaptoethanol: 20 μL, 0.2% v/v; DIPEA: 80 μL, 0.8% v/v; toluene: 9.4 mL, 94% v/v; duration of incubation: 24 h; total volume of reaction medium: 10 mL). (b) Effect of 2-mercaptoethanol composition (2−200 μL, 0.02−2.00% v/v) on the yield and dispersity of oligolysine in low-water toluene media (aqueous phase composition: 5%, 0.5 mL; L-Lys-OEt: 0.5 mmol, 50 mM; DIPEA: 80 μL, 0.8% v/v; toluene: 9.4 mL, 94% v/v; duration of incubation: 24 h; total volume of reaction medium: 10 mL). (c) Effect of DIPEA composition (8−800 μL, 0.08−8.00% v/v) on the yield and dispersity of oligolysine in low-water toluene media (aqueous phase composition: 5%, 0.5 mL; L-Lys-OEt: 0.5 mmol, 50 mM; 2-mercaptoethanol: 20 μL, 0.2% v/v; toluene: 9.4 mL, 94% v/v; duration of incubation: 24 h; total volume of reaction medium: 10 mL). (d) Effect of substrate concentration (0.5−3 mmol, 50−300 mM) on the yield and dispersity of oligolysine in low-water toluene media (aqueous phase composition: 5%, 0.5 mL; 2-mercaptoethanol: 20 μL, 0.2% v/v; DIPEA composition: 80 μL, 0.8% v/v; toluene: 9.4 mL, 94% v/v; duration of incubation: 24 h; total volume of reaction medium: 10 mL). (e) Effect of duration of incubation (0.5−24 h) on the yield and dispersity of oligolysine in low-water toluene media (aqueous phase composition: 5%, 0.5 mL; substrate concentration: 0.5 mmol, 50 mM; 2-mercaptoethanol: 20 μL, 0.2% v/v; DIPEA composition: 80 μL, 0.8% v/v; toluene: 9.4 mL, 94% v/v; total volume of reaction medium: 10 mL).

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the relative amounts of trilysine and tetralysine were lowered, with a stronger presence of pentalysine and hexalysine. This suggests that at water contents as low as 1−2%, the product peptides partition well into the toluene phase, thereby hindering the formation of higher-order oligolysines. As the water content increases, this partitioning is lowered, facilitating a greater aqueous phase residence time and higher-order oligolysine synthesis. As the water content was increased to 10%, the yield was also lowered to ∼45% with the dispersity ranging from 2 to 8 and beyond, but the predominant peaks in the sample were mainly pentalysine and hexalysine. This result suggests that though very high water content lowers the partitioning into the toluene phase and shifts the dispersity profile to higher-order oligolysine synthesis, it also lowers the yields due to the increasing dominance of the competing secondary hydrolysis reaction. Hence, an optimum water content of 5% v/v (0.5 mL in 10 mL of reaction mixture), which provides good yield and wide dispersity, was selected for all further reactions.

3.6.2. Effect of 2-Mercaptoethanol. Papain is a cysteine protease with a nucleophilic cysteine thiol in its active site. Oxidation of the thiol group will lead to a loss of catalytic activity of the papain. Hence, antioxidants such as 2-mercpatoethanol are essential components in such a reaction mixture which maintain the thiol groups of papain in the reduced state and also scavenge for any hydroxyl radicals. However, excess 2-mercaptoethanol is known to denature proteins and is widely used in gel electrophoresis to maintain proteins in their monomeric state. Hence, the amount of 2-mercaptoethanol was expected to be a likely factor in determining the yield and dispersity profile in papain-catalyzed oligolysine synthesis reactions. This was investigated by varying the amount of 2-mercpatoethanol in the 10 mL reaction mixture from 0 to 2% v/v while keeping all other variables constant, as listed in section 3.2. The processed sample was separated by ion-pair chromatography to identify the oligolysine peaks and record their area counts. These data were plotted as shown in Figure 5b and shows that the yield and dispersity vary considerably with the amount of 2-mercaptoethanol.

The absence of any 2-mercaptoethanol in the reaction mixture was found to lead to a zero yield, as its presence is considered essential for papain activity. When the amount of 2-mercaptoethanol was raised to 0.02%, the product mixture was found to contain trace amounts of dilysine and trilysine with very poor yield. As the amount of 2-mercaptoethanol was increased from 0.1% to 0.2%, the yield increased from ∼70% to a maximum of ∼85%, with the predominance of trilysine and tetralysine and the poor presence of higher-order oligolysines. An incremental increase of the amount of 2-mercaptoethanol to 1% provided a concomitant decrease of the yield to ∼70% and a shift in the range of dispersity to higher-order oligolysine products. A 2% 2-mercaptoethanol composition in the reaction mixture reduced the yield further to ∼10%. The poor yields at 0 and 0.1% reflect the significance of the reducing activity of 2-mercaptoethanol in the reaction. At higher 2-mercaptoethanol compositions, the amounts were most probably adequate for ensuring the activity of the papain, leading to yields as high as ∼85%. However, 2-mercaptoethanol levels above 1% led to poor yields, reflecting the toxic nature of the additive. This resulted in choosing 0.2% v/v 2-mercaptoethanol as the optimum concentration of all further experimental runs.[42]

3.6.3. Effect of DIPEA. *N*,*N*-Diisopropylethylamine is an organic base, also known as Hünig's base and often abbreviated as DIPEA in technical literature. It is also reported to have poor water solubility and high solubility in nonpolar solvents such as toluene. It has been reported to mediate amide synthesis reactions occurring through condensation of an acid and amine. The bulky propyl and ethyl groups in the midst of the amine moiety make it a poorly accessible nucleophile that can scavenge only protons and not compete with the amine of the amide (peptide) synthesis reaction. It can therefore be considered as an essential additive for peptide bond synthesis reactions in low-water organic media.[43] The effect of DIPEA on oligolysine yield and dispersity was investigated by varying its composition in the reaction medium from 0 to 8% v/v while maintaining the levels of all other ingredients constant, as described in section 3.2. The data were plotted in Figure 5c, which shows the effect of the composition of DIPEA on oligolysine yield and dispersity.

When the reaction mixture was composed of a DIPEA amount as low as 0.08%, the yield was almost nil with negligible presence of dilysine in the product, suggesting the crucial role of DIPEA in the synthesis reaction. As the amount of DIPEA was increased to 0.4%, the yield increased to ∼65%, with the product mixture composed of mainly dilysine, trilysine, and tetralysine. Further increases in the DIPEA amount to 0.8% and 2% led to the realization of ∼85% yield with the dispersity profile shifting toward the higher-order oligolysines tetralysine, pentalysine, hexalysine, and heptalysine. When the DIPEA amount was increased further to 4% and 8%, the yield was lowered to ∼60% and ∼10%, respectively, suggesting that high amounts of DIPEA were most probably toxic to the papain. These results put together helped in choosing 5% v/v aqueous phase, 0.2% v/v 2-mercaptoethanol and 0.8% v/v DIPEA as optimum amounts for maximizing the yield and realizing a wide dispersity of the product.

3.6.4. Effect of Substrate Concentration. In enzyme-catalyzed reactions, the substrate binds to the active site of the enzyme, lowering the activation energy needed for the reaction to proceed forward. At constant enzyme concentration, any increase in the substrate concentration leads to an increase in the reaction rate. This occurs until all the active sites on the enzymes are saturated, after which any further increase in substrate concentration does not lead to an increase in the reaction rate, which levels off. Hence, the enzyme/substrate (E/S) ratio may be considered as one of the factors determining the reaction rate. In the papain-catalyzed oligolysine synthesis in low-water organic media, this ratio may also be a factor that determines the rate of partitioning to the organic phase and subsequently the yield and dispersity of the product. To test this further, the substrate (L-Lys-OEt) concentration was varied from 50 to 300 mM (123 to 738 L-Lys-OEt·2HCl, respectively) in increments of 50 mM in the reaction medium while keeping the enzyme concentration constant. The E/S ratio in these experiments varied from 3.46 to 0.58% w/w, respectively. Figure 5d shows the effect of varying the substrate concentration on the yield and dispersity of oligolysine.

At 50 mM substrate concentration, an oligolysine yield of ∼85% was obtained, with the dispersity ranging from 2 to 8 residues and predominantly composed of trilysine and tetralysine. When the substrate concentration was increased to 100 mM, the oligolysine yield increased marginally to ∼90%

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as expected, with no change in the dispersity profile. As the substrate concentration was increased further, a deterioration in the oligolysine yield was observed with a narrower dispersity composed of mainly dilysine and trilysine. These results suggest that increasing the substrate concentration while keeping the enzyme concentration and the aqueous phase volume constant does not lead to any enhancement in the yield. These data suggest that 50 mM substrate concentration is adequately high enough to ensure high yield but with narrow oligolysine dispersity. The further increase in substrate concentration beyond 100 mM provided poor yield, suggesting that the very high amount of substrate concentrations in the aqueous part might hinder enzyme activity and be toxic to it.

3.6.5. Effect of Duration of Incubation. The papain-catalyzed synthesis of oligolysine with L-Lys-OEt in low-water toluene media is a kinetically controlled synthesis strategy. When the oligopeptide product remains in the aqueous phase, it is susceptible to secondary enzymatic hydrolysis and shortening of the chain. With sufficient duration of incubation and protease activity, the reaction will eventually reach equilibrium, where only L-lysine (free acid) monomers are dominant in the final mixture. Hence, in the kinetically controlled strategy, it is crucial to terminate the reaction when the product concentration is maximum. This can also be achieved by removing the product from the reaction medium, thereby preventing its accessibility for the hydrolysis reaction. The water-immiscible nonpolar solvent toluene used in this reaction system is assumed to play this role by effectively partitioning the oligopeptide chains away from the aqueous phase. During the course of the reaction, the enzyme also loses its activity due to unfavorable contact with the organic phase. Thus, beyond a certain duration of incubation, the secondary enzyme-catalyzed hydrolysis step is not effective for lowering the yield and dispersity of the oligopeptide. Hence, the duration of the incubation of the reaction mixture may also play a role in determining the eventual yield and dispersity of the oligolysine product. This was checked by varying the duration of incubation from 0.5 to 24 h, and the results of this are shown in Figure 5e.

When the duration of incubation was below 1 h, a maximum ∼30% yield was obtained, with the product composed of mainly dilysine peptides. As the duration of incubation was increased to 2 h, the yield was enhanced to ∼65%, with the product dispersity dominated mainly by dilysine and trilysine. An 8 h incubation period witnessed an increase in the yield to ∼80% with major presence of tetralysine and pentalysine in the product. Extending the period of incubation to 24 h extended the yield to ∼85%, suggesting that the papain was active beyond 8 h. This also led to the accumulation of tetralysine as the major component in the product at the cost of the pentalysine and higher-order homologues. This loss of wider dispersity was most probably due to the secondary hydrolysis. The OVAT analysis indicates that a maximum oligolysine yield of ∼85% was obtained with optimal values of 5% v/v water in toluene, 0.2% v/v 2-mercaptoethanol, 0.8% v/v DIPEA, 50 mM L-Lys-OEt, and 24 h incubation.

3.7. Statistical Analysis. *3.7.1. Optimization of Conditions for Oligolysine Synthesis with CCD and Analysis of Variance.* The OVAT methodology does not indicate the interactive effects between the variables, if any, affecting the response variable. This requires a statistical method such as a CCD methodology. CCD can also be used to (i) formulate a model equation that correlates the effect of significant variables on the oligolysine yield and (ii) determine the optimal values of the significant variables providing the maximum yield. The predominant variables selected in the PB design were also chosen for the CCD study. These variables and (in parentheses) their predetermined ranges used for the CCD study were the queous phase composition (A; 1−10%), 2-mercaptoethanol (B; 0.02−2% v/v), DIPEA (C; 0.08−8% v/v), substrate (L-Lys-OEt) concentration (D; 50−300 mM), and duration of incubation (E; 0.5−24 h). The stirring rate and temperature were maintained constant at 200 rpm and 25 °C in these experiments. The 50 runs suggested by the software and the corresponding response yield values obtained with the experiments are shown in Table 3.

The trial runs show that the response yield values range from 91.1% to 12.2%. The experimental data were subjected to multiple regression analysis, and a model expression having an R^2 value of 0.9348 was obtained as reproduced below:

$$\begin{aligned}\text{yield} = {} & 81.19 - 2.85\cdot A + 2.90\cdot B + 2.11\cdot C - 5.52\cdot D \\ & + 4.38\cdot E + 0.0531\cdot A\cdot B + 1.41\cdot A\cdot C + 1.83\cdot A\cdot D \\ & + 1.14\cdot A\cdot E - 1.65\cdot B\cdot C - 2.68\cdot B\cdot D + 0.9219\cdot B\cdot E \\ & + 2.35\cdot C\cdot D - 1.00\cdot C\cdot E - 0.6531\cdot D\cdot E - 3.71\cdot A^2 \\ & - 14.16\cdot B^2 - 24.16\cdot C^2 - 8.41\cdot D^2 - 3.21\cdot E^2\end{aligned}$$

This mathematical model, which is expressed in terms of the coded factors, can be used to predict the response. The factor coefficients in the expression can also be used to assess the relative impacts of these factors.

The ANOVA results, which examine the linear, quadratic, and interaction effects of variables and their p values, are tabulated in Table 4. The model F value obtained from the table is 20.78, which suggests that the model expression is significant. It also shows that there is only a 0.01% chance that this relatively high F value could occur merely due to noise. p values less than 0.05 can be used as a benchmark to indicate that the corresponding model expression terms are significant. In this case, D (substrate concentration), E (duration of incubation), B^2 (2-mercaptoethanol), and C^2 (DIPEA) are significant model terms strongly influencing the oligolysine yield. As a general rule for p values, $0.05 < p < 0.1$ and $p > 0.1$ may be used to designate the corresponding terms to be marginally significant and insignificant, respectively. The lack of fit F value obtained from the table is 9.09, which suggests that the lack of fit is significant. It also shows that there is only a 0.01% chance that this relatively large lack of fit F value could occur merely due to noise. The adequate precision, 12.9884, measures the signal-to-noise ratio. A ratio greater than 4 is desirable. This model can be used to navigate the design space.

3.7.2. Interaction Effects between Process Variables and Response (Yield). The CCD-derived 3D response surface plots are reproduced in Figure 6. They represent the interaction effects of pairs of process variables on the oligolysine yield, and each case is discussed below.

Figure 6a shows a 3D plot representing the interactive effects between the aqueous phase composition (A) and the 2-mercaptoethanol concentration (B) on the oligolysine yield.The aqueous phase composition and 2-mercaptoethanol concentration range from 1 to 10% and 0.02 to 2%, respectively. This is a simple-maximum-type surface plot with moderate curvature. This is also reflected in the contour trace lines separated by a relatively small interval of 5% yield values

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Table 3. Central Composite Design for Optimization of Parameters for Yield of Oligolysine Product (A: Aqueous Phase Ratio; B, 2-Mercaptoethanol; C: DIPEA; D: Substrate Concentration; E: Duration of Incubation)

run order	A (%)	B (%)	C (%)	D (mM)	E (h)	yield (%)
1	5.5	1.01	4.04	300	12.25	45.3
2	1.0	2.00	0.08	300	24.00	23.2
3	10	2.00	8.00	50	24.00	42.3
4	1.0	2.00	8.00	300	24.00	28.1
5	5.5	1.01	4.04	175	12.25	84.2
6	10.0	1.01	4.04	175	12.25	62.6
7	10.0	2.00	8.00	300	0.50	19.2
8	1.0	0.02	0.08	50	24.00	31.1
9	10.0	0.02	8.00	50	24.00	29.9
10	5.5	1.01	4.04	175	12.25	82.5
11	5.5	0.02	4.04	175	12.25	53.8
12	1.0	2.00	8.00	50	24.00	41.2
13	10.0	0.02	0.08	300	24.00	15.6
14	10.0	0.02	8.00	50	0.50	17.9
15	1.0	0.02	8.00	50	24.00	34.2
16	10.0	0.02	0.08	50	24.00	24.9
17	1.0	0.02	0.08	300	24.00	20.2
18	5.5	1.01	4.04	175	12.25	88.2
19	1.0	0.02	8.00	50	0.50	29.6
20	5.5	1.01	4.04	175	12.25	90.5
21	5.5	1.01	4.04	50	12.25	86.2
22	5.5	1.01	4.04	175	0.50	51.6
23	1.0	0.02	8.00	300	24.00	33.4
24	5.5	1.01	4.04	175	12.25	88.7
25	1.0	2.00	8.00	50	0.50	34.2
26	1.0	2.00	8.00	300	0.50	22.6
27	10.0	0.02	8.00	300	0.50	27.8
28	5.5	1.01	4.04	175	24.00	90.3
29	10.0	0.02	0.08	300	0.50	12.2
30	10.0	2.00	8.00	300	24.00	32.2
31	5.5	1.01	4.04	175	12.25	89.5
32	10.0	0.02	8.00	300	24.00	33.1
33	5.5	1.01	4.04	175	12.25	91.1
34	1.0	2.00	0.08	50	0.50	44.2
35	10.0	2.00	8.00	50	0.50	27.4
36	1.0	2.00	0.08	50	24.00	51.1
37	10.0	0.02	0.08	50	0.50	20.1
38	1.0	2.00	0.08	300	0.50	20.6
39	1.0	0.02	0.08	50	0.50	29.8
40	10.0	2.00	0.08	50	24.00	38.6
41	10.0	2.00	0.08	50	0.50	24.5
42	5.5	2.00	4.04	175	12.25	66.2
43	5.5	1.01	0.08	175	12.25	44.2
44	10.0	2.00	0.08	300	0.50	17.7
45	5.5	1.01	8.00	175	12.25	55.8
46	1.0	0.02	8.00	300	0.50	24.5
47	1.0	0.02	0.08	300	0.50	20.1
48	1.0	1.01	4.04	175	12.25	78.3
49	5.5	1.01	4.04	175	12.25	91.0
50	10.0	2.00	0.08	300	24.00	23.6

and projected on the floor of the plot. The plot shows that at a constant 1% phase composition, as the 2-mercaptoethanol concentration was increased from 0.02% to 1%, the oligolysine yield was found to increase from a low of ∼58% and attain a maximum of ∼75%, beyond which the yield started to gradually decrease and attain a value of ∼65% at 2% 2-mercaptoethanol concentration. A similar trend is observed at higher phase compositions from 1 to 10%. The data also suggest that 0.02% 2-mercaptoethanol concentration is not adequate enough to act as a reducing agent and maximize the oligolysine yield. In fact, experiments in the absence of 2-mercaptoethanol provided poor oligolysine, suggesting that the reducing agent is crucial to maintain papain activity. The results show that 2-mercaptoethanol concentrations above 1% lead to a reducing trend of oligolysine yield, and this is most probably due to the fact that the higher concentrations induced toxicity to the papain activity. These results are in concurrence with the discussions about the effect of 2-mercaptoethanol made in section 3.6.2. The discussions about the role of the aqueous phase composition in low-water organic media given in section 3.6.1 can be used to make the following deductions. Very low aqueous phase compositions are unfavorable for oligolysine synthesis, and this is most probably due to a combination of two factors: (i) inadequate reaction volume leading to low water activity for the enzyme to sustain its activity and (ii) higher organic to aqueous phase ratio enhancing the partitioning of the growing peptide chain into the organic phase and lowering the residence time in the aqueous phase. At the other extreme, at very high aqueous phase compositions, the yield is again low, most probably because a low organic to aqueous phase ratio promotes (i) higher water activity and residence time in the aqueous phase and (ii) poorer partitioning of the growing peptide chain to the organic phase, leading to poor conversions and the hydrolysis of the product peptides. The results of the 3D surface and contour plot put together show that as the aqueous phase composition is increased from 1 to 10% v/v at constant 0.02% v/v 2-mercaptoethanol concentration, the oligolysine yield initially increased from a low of ∼58% and attained a maximum of ∼60% at ∼3−4% aqueous phase composition, followed by a decrease to ∼58%. This almost flat response trend is observed for all 2-mercaptoethanol compositions studied. The 3D surface plot and contour traces in Figure 6a show that the oligolysine yield can be maximized at ∼1% v/v 2-mercaptoethanol and ∼3−4% v/v aqueous phase composition in the low-water toluene reaction medium. The relatively flat nature of the quadratic curve also can be used to infer marginal interactive effects between the aqueous phase compositions and 2-mercaptoethanol concentrations investigated.

The 3D surface plot in Figure 6b depicts the interactive effects between aqueous phase composition (A, 1−10% v/v) and DIPEA composition (C, 0.08−4% v/v) against oligolysine yield in the low-water organic media. The surface plot and the contour trace lines show that this is a maximum-type curve with a moderate positive curvature. The figure shows that when the aqueous phase composition was held constant at 1%, the yield increased from ∼54% at 0.08% DIPEA to a maximum of ∼70% at ∼4% DIPEA. Further increase in the DIPEA composition led to a deterioration in the yield, attaining a low of ∼56% yield at 8% DIPEA. This trend is evident at all aqueous phase compositions ranging from 1 to 10% as shown in the figure. As discussed in section 3.6.3, DIPEA is reported to be an essential additive in such reactions, as it neutralizes the S1 subsite of papain for cationic substrates such as lysine.[39] It is also been reported to scavenge protons and not hinder peptide bond synthesis reactions.[43] Hence, a minimum concentration of DIPEA might be necessary for peptide synthesis in such low-water organic media. This most probably

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Table 4. ANOVA Results of the CCD Optimization of Parameters Affecting the Yield of Oligolysines (A: Aqueous Phase Ratio; B: 2-Mercaptoethanol; C: DIPEA; D: Substrate Concentration; E: Duration of Incubation)

source model	sum of squares	degrees of freedom	mean square	F	p
model	30503.76	20	1525.19	20.78	<0.0001
A (%)	275.6	1	275.6	3.76	0.0624
B (%)	286.52	1	286.52	3.9	0.0577
C (%)	151.2	1	151.2	2.06	0.1619
D (mM)	1037.32	1	1037.32	14.14	0.0008[a]
E (h)	652.97	1	652.97	8.9	0.0057[a]
AB	0.0903	1	0.0903	0.0012	0.9723
AC	63.56	1	63.56	0.8661	0.3597
AD	106.95	1	106.95	1.46	0.2371
AE	41.63	1	41.63	0.5673	0.4574
BC	86.79	1	86.79	1.18	0.2858
BD	229.52	1	229.52	3.13	0.0875
BE	27.2	1	27.2	0.3706	0.5474
CD	177.19	1	177.19	2.41	0.1311
CE	32.2	1	32.2	0.4388	0.5129
DE	13.65	1	13.65	0.186	0.6695
A^2	34.06	1	34.06	0.4641	0.5011
B^2	495.97	1	495.97	6.76	0.0145[a]
C^2	1443.8	1	1443.8	19.67	0.0001[a]
D^2	174.97	1	174.97	2.38	0.1334
E^2	25.5	1	25.5	0.3474	0.5601
residual	2128.21	29	73.39		
lack of fit	2056.24	22	93.47	9.09	0.003
pure error	71.97	7	10.28		
cor total	32631.97	49			

[a]Significant model terms ($p < 0.05$).

provides the rationale for poor yields with low DIPEA concentrations and further increase in DIPEA leading to a maximum yield at ∼4% concentration, as shown in Figure 6b. However, any subsequent increase in DIPEA concentration was observed to lead to a degradation of the yield, most probably due to its toxic effects on papain activity. Figure 6b also shows that when the DIPEA concentration is held constant at 0.08% and the aqueous phase composition is increased from 1 to 10%, the yield increases from ∼54% to a maximum of ∼70% at ∼3−4% DIPEA but deteriorates beyond that, with a ∼47% yield at 10% aqueous phase composition. This trend is reflected at all DIPEA concentrations, leading to the contour plot shown in Figure 6b. The contour traces of Figure 6b suggest that a maximum oligolysine yield of ∼85% can be obtained with ∼4% v/v DIPEA concentration and ∼3−4% v/v aqueous phase composition in the low-water toluene medium. The plot can also be used to infer that a moderately significant interaction exists between the aqueous phase and DIPEA compositions.

The interactive effects between aqueous phase composition (A, 1−10% v/v) and substrate (L-Lys-OEt) concentration (D, 50−300 mM) affecting the oligolysine yield are shown in Figure 6c. The 3D surface plot is a simple maximum-type curvature with what appears to be an almost planar-type surface. The contour traces represent lines from ∼65% to ∼80% yield in intervals of 5% yield each. The plot shows that when the aqueous phase composition is held constant at its lowest value studied (i.e., 1% v/v), increasing the substrate concentration from 50 to 200 mM did not affect the ∼85% yield, but a reduction to ∼62% was observed at 300 mM. A similar trend was observed at all aqueous phase compositions ranging to 10%. The enzyme to substrate ratio is one of the critical factors affecting enzyme kinetics and product yield in kinetically controlled reactions, as discussed in section 3.6.4. The results from this trend show that at 1% v/v aqueous phase composition, a 50 mM substrate concentration was more than adequate to realize a yield as high as ∼85%. As the range of concentrations was not extended below 50 mM, it is difficult to extrapolate the trend and determine a concentration providing higher yield. However, the contour traces obtained from this plot can be used to conclude that 50 mM L-Lys-OEt concentration and ∼3−4% v/v aqueous phase composition provided the maximum oligolysine yield of ∼85%. The relatively planar nature of the surface plot and the closely spaced contour traces show that poor significance can be attached to the combined interaction of aqueous phase composition and substrate concentration on the oligolysine yield.

The interaction between the duration of incubation (E) ranging from 0.5 to 24 h and aqueous phase composition (A) ranging from 1 to 10% v/v and their effect on the oligolysine yield is illustrated in Figure 6d. As discussed in section 3.6.5, the duration of the incubation is a decisive factor in kinetically controlled reactions. During the initial phase of the reaction, the oligolysine yield is expected to increase rapidly along with the partitioning of the product oligopeptides to the organic phase. Hence, in these reactions, extending the reaction duration beyond the duration when maximum oligopeptide products appear to be present in the medium might lead to the reverse hydrolysis reaction to form lysine free acid monomers. However, this is concomitant with the enzyme losing its activity gradually due to the unfavorable contact with the organic medium, rendering the secondary enzymatic hydrolysis step ineffective. The 3D plot in Figure 6d is similar to a simple

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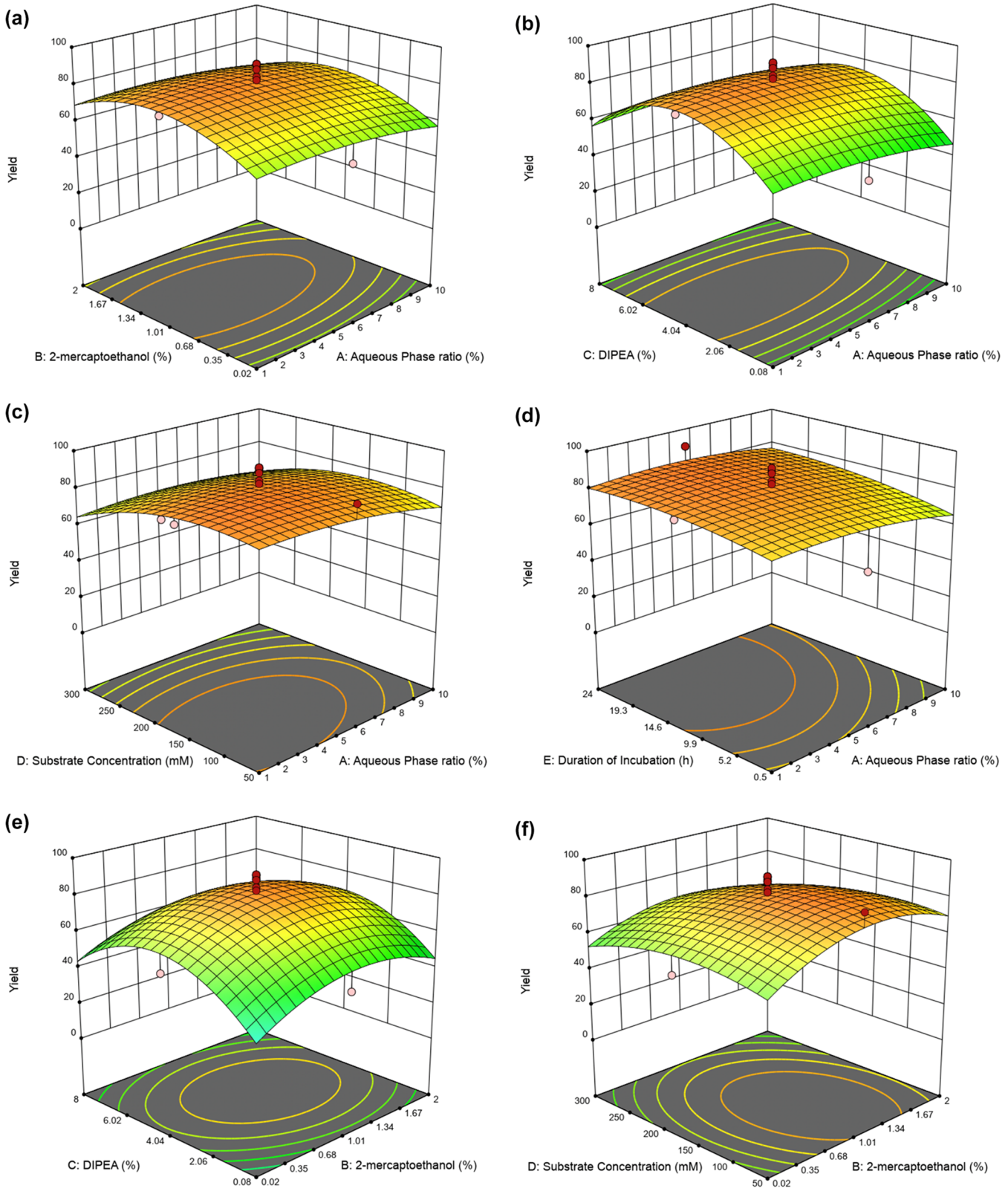

Figure 6. continued

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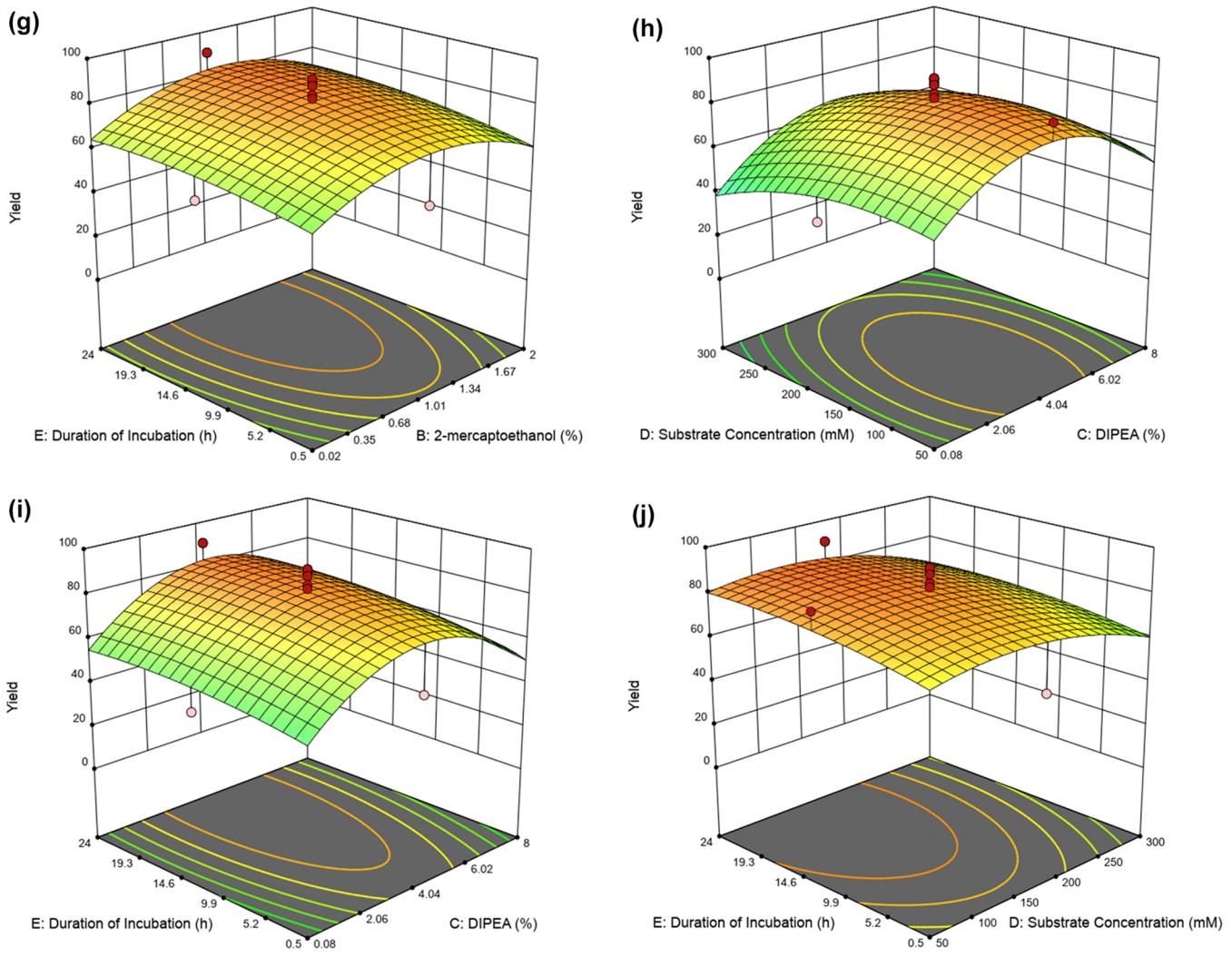

Figure 6. (a) 3D surface plot and contour plot showing interactions between (A) aqueous phase composition and (B) 2-mercaptoethanol (substrate concentration: 0.5 mmol, 50 mM; DIPEA composition: 80 μL, 0.8% v/v; toluene: 9.4 mL, 94% v/v; duration of incubation: 24 h; total volume of reaction medium: 10 mL). (b) 3D surface plot and contour plot showing interactions between (A) aqueous phase composition and (C) DIPEA (substrate concentration: 0.5 mmol, 50 mM; 2-mercaptoethanol: 20 μL, 0.2% v/v; toluene: 9.4 mL, 94% v/v; duration of incubation: 24 h; total volume of reaction medium: 10 mL). (c) 3D surface plot and contour plot showing interactions between (A) aqueous phase composition and (D) substrate concentration (2-mercaptoethanol: 20 μL, 0.2% v/v; DIPEA composition: 80 μL, 0.8% v/v; toluene: 9.4 mL, 94% v/v; duration of incubation: 24 h; total volume of reaction medium: 10 mL). (d) 3D surface plot and contour plot showing interactions between (A) aqueous phase composition and (E) duration of incubation (substrate concentration: 0.5 mmol, 50 mM; 2-mercaptoethanol: 20 μL, 0.2% v/v; DIPEA composition: 80 μL, 0.8% v/v; toluene: 9.4 mL, 94% v/v; total volume of reaction medium: 10 mL). (e) 3D surface plot and contour plot showing interactions between (B) 2-mercaptoethanol and (C) DIPEA (aqueous phase composition: 5%, 0.5 mL; substrate concentration: 0.5 mmol, 50 mM; toluene: 9.4 mL, 94% v/v; duration of incubation: 24 h; total volume of reaction medium: 10 mL). (f) 3D surface plot and contour plot showing interactions between (B) 2-mercaptoethanol and (D) substrate concentration (aqueous phase composition: 5%, 0.5 mL; DIPEA composition: 80 μL, 0.8% v/v; toluene: 9.4 mL, 94% v/v; duration of incubation: 24 h; total volume of reaction medium: 10 mL). (g) 3D surface plot and contour plot showing interactions between (B) 2-mercaptoethanol and (E) duration of incubation (aqueous phase composition: 5%, 0.5 mL; substrate concentration: 0.5 mmol, 50 mM; DIPEA composition: 80 μL, 0.8% v/v; toluene: 9.4 mL, 94% v/v; total volume of reaction medium: 10 mL). (h) 3D surface plot and contour plot showing interactions between (C) DIPEA and (D) substrate concentration (aqueous phase composition: 5%, 0.5 mL; 2-mercaptoethanol: 20 μL, 0.2% v/v; toluene: 9.4 mL, 94% v/v; duration of incubation: 24 h; total volume of reaction medium: 10 mL). (i) 3D surface plot and contour plot showing interactions between (C) DIPEA and (E) duration of incubation (aqueous phase composition: 5%, 0.5 mL; substrate concentration: 0.5 mmol, 50 mM; 2-mercaptoethanol: 20 μL, 0.2% v/v; toluene: 9.4 mL, 94% v/v; total volume of reaction medium: 10 mL). (j) 3D surface plot and contour plot showing interactions between (D) substrate concentration and (E) duration of incubation (aqueous phase composition: 5%, 0.5 mL; 2-mercaptoethanol: 20 μL, 0.2% v/v; DIPEA composition: 80 μL, 0.8% v/v; toluene: 9.4 mL, 94% v/v; total volume of reaction medium: 10 mL).

maximum type but possesses a poor curvature and appears almost like a plane surface. The figure shows that at a constant 1% aqueous phase composition, when the duration of incubation was increased from 0.5 to 24 h, the oligolysine yield was enhanced from ~70% to 80%. When the aqueous phase composition is held constant at much higher 10% v/v value, a similar trend is observed with an increase in yield from ~58% at 0.5 h to ~75% at 24 h incubation. The contour traces extending from 60% to 80% in intervals of 5% yield can be used to infer that extending the duration of incubation leads to

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a very gradual increase in the yield, but this is most effective at low aqueous phase compositions. These results are in concurrence with the discussions about the effect of the duration of incubation made in section 3.6.5. The contour traces show that a maximum yield of ∼80% can be obtained with ∼20 h incubation and ∼3−4% v/v aqueous phase composition. The surface and contour plots can be used to conclude that a relatively poor correlation exists between aqueous phase composition and duration of incubation. Figure 6a−e shows the effect of aqueous phase composition (A) on 2-mercaptoethanol (B), DIPEA (C), substrate concentration (D), and duration of incubation (E), respectively. The 3D plots show that in the aqueous phase composition range investigated (1−10%), the most a marginal significance can be attributed to its interaction with other variables. This suggest that overall the aqueous phase composition, though contributing to the high yields observed, does not to appear to depend on the other significant variables.

The significance of the two additives, 2-mercaptoethanol (B) and DIPEA (C), has been discussed in sections 3.6.2 and 3.6.3, respectively. They are known to be effective at low concentrations, but their toxicity lowers the yield and dispersity at higher concentrations. The synergistic effect of these additives on oligolysine yield is represented in Figure 6e, which can be termed as a positive-maximum-type response surface. When the 2-mercaptoethanol concentration is held constant at 0.02% v/v and DIPEA concentration is varied from 0.08 to 8% v/v, the yield which is low at ∼35% increases until it attains a maximum of ∼70% at ∼4% DIPEA. This maximum at ∼4% DIPEA trend was observed across all values of the 2-mercaptoethanol concentration studied from 0.02 to 2% v/v. A similar trend was observed while varying the 2-mercaptoethanol between 0.2 and 2% v/v with a maximum at 1% v/v 2-mercaptoethanol. The contour projection from this plot can be used to conclude that a maximum yield of ∼80% can be obtained with ∼1% v/v and ∼4% v/v 2-mercaptoethanol and DIPEA concentrations, respectively. The plots can also be used to infer that a strong interactive effect exists between the two variables.

Figure 6f portrays the mutually dependent effect of the additive 2-mercaptoethanol (B) and the substrate L-Lys-OEt (D) concentrations on the oligolysine yield. The 3D surface response is shaped as a simple-maximum-type curve, with the contour lines ranging from 50% to 90% yield. When the 2-mercaptoethanol concentration was maintained at 0.02% v/v, the oligolysine yield was observed to be ∼58% at 50 mM, pass through at maximum of ∼65% at 100 mM, and finally decrease to ∼45% at 300 mM, the maximum substrate concentration utilized in the CCD. As observed in Figure 6c with aqueous phase composition, the maximum yield was obtained with 100 mM L-Lys-OEt for the complete range of 2-mercaptoethanol concentrations investigated (0.02−2% v/v). At the 50 mM concentration, the 0.02% and 2% v/v 2-mercaptoethanol concentrations produced ∼58% and ∼75% yield, respectively, with a maximum of ∼80% yield at ∼1.3% v/v 2-mercaptoethanol concentration. This maximum at ∼1.3% v/v 2-mercaptoethanol concentration was observed to occur at all substrate concentrations studied (50 to 300 mM). These contour plot results show that a ∼80% maximum oligolysine yield can be realized at the optimum 2-mercaptoethanol and substrate concentrations of ∼1.3% v/v and 100 mM, respectively. The plot also shows that the two variables, the 2-mercaptoethanol and L-Lys-OEt concentrations, mutually interact with each other to affect the oligolysine yield.

The combined effect of varying the 2-mercaptoethanol concentration (B, 0.02−2% v/v) and duration of incubation (E, 0.5−24 h) on oligolysine yield is illustrated in Figure 6g. The 3D surface has a simple maximum profile with contour lines extending from 50% to 90% yield. Figure 6g shows that with a 2-mercaptoethanol concentration of 0.002% v/v, the yield increased from ∼46% at 0.5 h to attain a ∼58% maximum at 12 h, which did not change for any further period of incubation. On the other extreme, with a 2% v/v 2-mercaptoethnaol concentration, in the first 0.5 h incubation period, a yield as high as 56% was obtained, which increased to 75% beyond 12 h incubation period. On the contrary, with a steady 0.5 h incubation, the yield varied between ∼46% and ∼58% for 0.02% and 2% 2-mercaptoethanol, respectively. This was accompanied with a maximum at ∼78% at 1% v/v 2-mercaptoethanol concentration. At higher incubation periods, the maximum yield increased to plateau at ∼90% with 1% 2-mercaptoethanol and ∼5 h incubation. The contour line characteristics were used to deduce that only a moderate interactive effect was observable for the 2-mercaptoethanol concentration and duration of incubation. Figure 6e−g put together characterizes the effect of 2-mercaptoethanol (B) on DIPEA concentration (C), substrate concentration (D), and duration of incubation (E), respectively. To summarize, 2-mercaptoethanol has a strong reciprocal effect with DIPEA, while it has a marginal correlation with the substrate and duration of incubation on oligolysine yields.

Figure 6h−j shows 3D response surface plots for oligolysine yield against different pairs of significant variables: DIPEA concentration (C) versus substrate concentration (D); DIPEA concentration (C) versus duration of incubation (E); and substrate concentration (D) versus duration of incubation (E), respectively. Figure 6h with a simple-maximum-type curve shows that when the DIPEA is held constant at 0.08% v/v and the substrate concentration is varied from 50 to 300 mM, the oligolysine yield is observed to decline steadily from ∼55% to ∼38% with a maximum at 100 mM substrate concentration. This trend was reproduced for all of the DIPEA concentrations studied (up to 8% v/v), suggesting that 50 and 100 mM substrate concentrations studied were optimum for maximizing oligolysine yield. At a steady substrate concentration of 50 mM, when the DIPEA was varied between 0.08 to 8% v/v, the oligolysine yield profile exhibited a maximum of ∼80% corresponding to ∼4% v/v DIPEA. The contour projection for this plot was used to interpret that (i) the maximum yield can be realized with ∼100 mM L-Lys-OEt and ∼4% v/v DIPEA concentrations and (ii) there is a strong correlative effect of the two variables on the yield.

Figure 6i is similar to the previous surface response plots and illustrates that when the DIPEA concentration is held at its lowest value studied, 0.02% v/v, the yield starts off at ∼45% at 0.5 h and plateaus at ∼55% beyond the 15 h incubation period. The same trend was obtained with the 8% v/v DIPEA concentration, from ∼45% yield at 0.5 h to ∼55% at 15 h and beyond. When the duration of incubation was held constant and the DIPEA concentration was varied between 0.08 and 8%, the yield increased to attain a maximum at ∼4% v/v DIPEA concentration. The 3D plot was used to predict a maximum ∼90% yield at around 24 h incubation. The contour and 3D plots put together show that the DIPEA concentration

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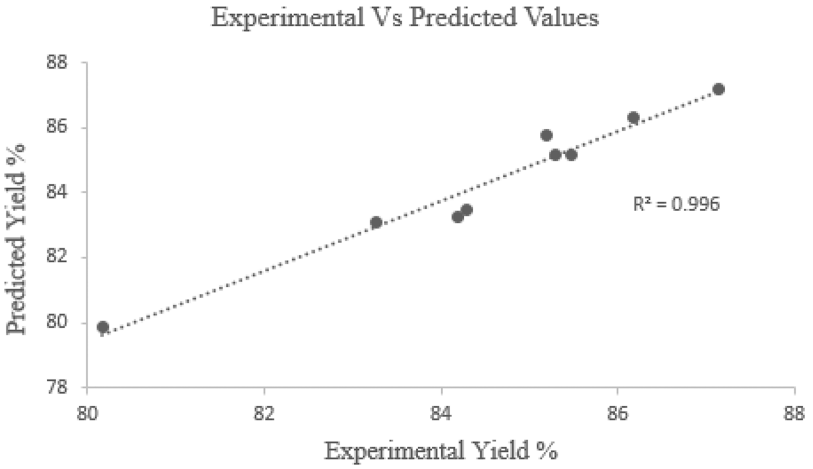

Figure 7. Predicted vs experimental oligolysine yield plot with correlation values to deduce the validity of the model.

has a significant interactive effect with the duration of incubation to affect the yield.

The last interactive effect predicted by the CCD study is the substrate concentration (D) with the duration of incubation (E), as depicted in Figure 6j. When the substrate concentration was held constant at 50 mM and the duration of incubation varied from 0.5 to 24 h, the yield was found to increase gradually from 72% to 82%, with the same trend observed for all substrate concentrations. However, when the substrate concentration was varied from 50 to 100 mM, the yield at all durations of incubation showed a small increase from 50 to 100 mM followed by a gradual decrease. This result along with the relatively flat 3D surface and broad contour lines shows that substrate concentration and duration of incubation have at the most a marginal interactive effect. The optimum conditions for the maximum predicted yield of ∼90% were ∼24 h of incubation and 100 mM substrate concentration. Figure 6 can be used to interpret that 2-mercaptoethanol and DIPEA appear to have the most significant interactive effect on oligolysine yield with the aqueous phase composition, substrate concentration, and duration of incubation.

3.8. Model Verification and Scale-Up Studies. The statistical Design of Experiments (DoE) has been used on a laboratory scale to assess multiple input variables simultaneously for their individual as well as interactive effects on the desired product attributes, the yield and product dispersity in this case. The validity of the DoE-derived polynomial model expression needs to be tested by checking its ability to predict the response function. This was tested by plotting the predicted oligolysine yield value against the experimentally obtained value as obtained from the software and is shown in the Figure 7. The regression value from this plot, R^2 = 0.996, can be used as measure of the correlation between the predicted and experimental values. The strong correlation can be used to conclude that the polynomial model expression is a good representation of the effect of the variables on the yield.

Any process development efforts should culminate in successful process scale-up. The feasibility of scale-up of the developed experimental synthesis model was investigated with a 10× upscaling setup. The reaction was conducted as a one-pot synthesis setup in a 250 mL flat-bottom flask with a magnetic stirrer at room temperature. Around 1.23 g of L-lysine-OEt·2HCl (5 mol, 50 mM) was weighed into the flat-bottom flask along with the addition of 94 mL of toluene, 200 μL of 2-mercaptoethanol, and 800 μL of DIPEA. The 5 mL aqueous phase bearing the ∼30 mg of papain as a suspension was added in the end to initiate the reaction. At the end of the 24 h incubation period, the contents were dried in a rotary evaporator, resuspended in water, and analyzed on HPLC to determine the oligolysine yield and dispersity. The results (not shown) of this preliminary investigation show that oligolysine is synthesized at these scaled-up conditions also in ∼75% yield with the number of residues ranging from 2 to 7. These efforts show that scale-up can be performed for such novel microaqueous organic media-driven reactions. However, as mentioned, this was only a preliminary investigation, and further experiments are necessary to identify the process parameters that need to be measured and controlled for designing peptides with desired yield and dispersity.

4. CONCLUSIONS

Polycationic compounds, such as basic amino acid peptides, have been reported to have many scientific and technical applications. Some literature also shows that the biological activity of these polycationic amino acid peptides depends on the degree of polymerization. Hence, there is a need for developing methods for synthesizing oligopeptides with the desired residue profile in high yields. Enzymatic methods are facile, devoid of byproducts, economical, and environmentally friendly and have been widely employed for biosynthesis and biotransformation reactions. Proteases have been shown to catalyze the synthesis of oligopeptides in aqueous buffered media with activated amino acids in their esterified form. With hydrophobic amino acids, the degree of polymerization is limited by the precipitation of the growing hydrophobic peptide chain. With hydrophilic amino acids such as lysine, the growing oligolysine chains do not precipitate, continue to remain in solution, and are subjected to proteolytic secondary hydrolysis, leading to poor yields and dispersity at equilibrium. This can be prevented by limiting the water activity in the reaction medium. A low-water organic medium consisting of a microaqueous phase in contact with bulk toluene providing low water activity has been utilized in this work to demonstrate the papain-catalyzed oligolysine production. The organic medium was also expected to extract the oligolysine product away from papain in the aqueous phase, preventing the secondary hydrolysis. A probable mechanism for the papain-catalyzed peptide chain initiation, elongation, and termination

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steps was also proposed. The identity of the individual oligolysine ion-pair LC peaks was established with semi-preparatory LC peak collection, followed by SPE purification and LC-MS analysis. The Plackett−Burman design was used to screen for significant variables. The five shortlisted variables based on a confidence level of 95% and R^2 value of 93.39% were aqueous phase composition, 2-mercaptoethanol concentration, DIPEA concentration, duration of incubation, and substrate concentration. The effect of varying the magnitudes of these variables on the yield and dispersity was investigated with the one-variable-at-a-time method. The results also show that low aqueous phase composition of 1−5% v/v, optimum 2-mercaptoethanol and DIPEA additive concentrations of 0.5−1% v/v and 2−4% v/v respectively, greater than 15 h incubation, and 50−100 mM substrate concentrations promote good yields (∼80 to 85%) with wide lysine residue dispersity (2−10). Statistical analysis with CCD methodology was also used to study the interactive effect of these significant variables and obtain a model expression which was validated with an R^2 value of 0.966. The interactive effects studied with the 3D surface and contour plots show that 2-mercaptoethanol and DIPEA concentrations have a significant effect on the other variables and that their concentrations are strong determinants of the yield and dispersity of the oligolysine product.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.oprd.3c00198.

MALDI-ToF analysis spectrum of oligolysine synthesis product mixture (PDF)

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Notes

The authors declare no competing financial interest.

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