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INFLUENCE OF pH ON THE ACYLATION OF L-HYDROXYPROLINE WITH PALMITOYL CHLORIDE IN AN AQUEOUS ACETONE MEDIUM

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ABSTRACT

Acylation reactions under Schotten-Bauman condition depend largely on the pH of the reaction medium. When the pH of the reaction medium was 9.0-10.0, the acylation of 0.1 mol of hydroxyproline with 2.5 mol of palmitoyl chloride in an aqueous acetone medium gave O*-palmitoyl hydroxyproline and palmitic acid. In another reaction at a pH of 10.5-13.0, a small portion of the formed* O*-palmitoyl hydroxyproline was converted into* N,O*-di-palmitoyl hydroxyproline which is only between the range of 4.79% to 10.78%. However, at more higher pH values, the* N*-acylation reaction competed with hydrolysis of the formed* O*-palmitoyl hydroxyproline to regenerate palmitic acid and L-hydroxyproline which resulted in the reduction of percentage of* N,O*-di-palmitoyl hydroxyproline of 9.00%.*

Keywords: acylation, Schotten-Bauman conditions, amino acid, palmitic acid, palm oil.

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INTRODUCTION

Acylation of amino acids by attachment of hydrocarbon molecules occurs through nucleophilic attack at carbonyl carbon of the acylating agent. The Schotten-Bauman procedure, which involves acylation of N-terminal amino acids with acyl chlorides in alkaline aqueous solutions are carried out at pH just slightly above the pKa value of the amino group (Kemp and Vellacio, 1980). Acid chlorides have been widely used as acylating agents in most acylation reactions involving introduction of long chain fatty acid to the amino part of amino acids (Xia *et al*., 2001). However, new approaches to improve or activate acyl chlorides for the acylation of nucleophiles of a weaker electronegativity are perpetually under development (Wakeham *et al*., 2013). Numerous attempts and innovative procedures were developed for selective acylation of amino acids with multiple nucleophilic sites. Acylation of lysine, an amino acid with α- and ε-amino groups, gave mixtures of its di-acyl and mono-acyl products of α- and ε-amino groups because of the similar reactivity of these nucleophiles (Denommee, 2007). Mali *et al*. (2013) investigated the selective *N*-acylation of amines through the thio acid-mediated reactions in methanol under neutral conditions. The *N*-acylation of amino acids predominates over *O*-acylation of aliphatic hydroxyl group in the amino acids serine and threonine in an aqueous medium (Xia *et al*., 2001).

Studies on the behaviour of acylation reactions that require all the different nucleophiles to be acylated are limited. In most cases, methods have been developed to selectively acylate the

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nucleophilic N of an amino acid. The amino acid L-hydroxyproline, for example, is well-known for its collagen cell renewal properties (Anon). However, L-hydroxyproline requires some degree of hydrophobicity so that it can be trans-dermally transported into the dermis layer of the skin when applied topically and functions as an anti-aging agent (Barton *et al*., 2011; Denommee, 2007). The L-Hydroxyproline has two nucleophilic sites: a pyrrolidine amino group and a pyrrolidin-3-ol hydroxyl group. Both of these nucleophiles need to be acylated, thus producing *N,O*-di-acylated hydroxyproline. However, because of the difference in electronegativity between these two groups, mixtures of acylated products are expected during acylation using Schotten-Bauman procedures. The elucidation of the behaviour of this reaction under different conditions is important to optimise the yield of *N,O*-di-palmitoyl hydroxyproline.

Generally, the hydrocarbon moiety of an acylating agent to be used to acylate amino acids for skin care products is the palmitoyl moiety because it is one of the most compatible hydrocarbon moieties found in the skin. Palmitic acid is a major component of palm oil (42%), along with oleic and linoleic acid (41% and 10%, respectively) (Rupillius and Ahmad, 2007).

Numerous inventions related to the use of di-palmitoyl hydroxyproline as a key ingredient in skin-care compositions have been developed (Mc Nulty and Rhodes, 2005; Dorf, 2007; Pinnell, 2007). The content of *N,O*-di-palmitoyl hydroxyproline in such products may vary depending on the process adopted for the production of the *N,O*-di-palmitoyl hydroxyproline. According to the study on kinetics of acylation reaction conducted by Kuritsyn *et al*. (2003) the reaction rate constant of the solvent mediated acylation reaction of amino acid glycine and proline with 4-nitrophenyl-4-nitrobenzoate increased with the increase in the aqueous content of the reaction medium. In addition, a study by Takehara *et al*. (1974) revealed that, besides reaction conditions, composition of the solvent medium and carbon chain length of the acylating agent play a very important role in optimising the yield of an acylating reaction. Therefore, the objective of our study is to investigate the influence of the assigned pH range of an aqueous acetone medium on the affinity of palmitoyl chloride with L-hydroxyproline in a fixed composition of aqueous acetone medium.

EXPERIMENTAL METHOD

Materials

The L-hydroxyproline, palmitoyl chloride and solvents were purchased from Sigma Aldrich (M) Sdn Bhd, Malaysia and used without further purification. Solutions of 1 N NaOH and 1 N HCl were prepared for neutralisation and acidification steps, respectively.

General Procedures for Acylation Reactions

An aqueous solution of L-hydroxyproline was made by dissolving 13.1 g (0.1 mol) in 100 ml distilled water in a 500 ml-jacketed glass beaker. The pH of the solution was adjusted to 10.0 by addition of about 10 ml of 1N NaOH solution. In another container, 68.7 g (0.25 moles) of palmitoyl chloride was carefully dissolved in 50 ml acetone and placed in a 100 ml-addition funnel equipped with glass stopper and pressure equaliser mounted on a retort stand. Two 50 ml-burettes were filled up with 1 N NaOH and 1 N HCl solutions, respectively. The palmitoyl chloride solution was alternately added drop wise into the L-hydroxyproline solution so that the pH of the reaction mixture was maintained at the assigned pH range throughout the addition procedure, while the temperature of reaction was controlled at 15.0°C. The mixture was allowed to stand while stirring at 15.0°C for another 4 hr and then allowed to stand at room temperature overnight or until a thick white cloudy dispersion was formed. The cloudy dispersion was then titrated with 1 N HCl solution until the pH of the solution dropped down to about 1.0. The floating white solid product was then separated from the clear aqueous-acetone phase through filtration and then washed with deionised water. The solid crude product mixture was subsequently dried in an oven at incremental increase of temperature (70°C-100°C) to remove potential volatiles and stored in a desiccator. The range of pH was regulated by alternating the titration of palmitoyl chloride and 0.1 N sodium hydroxide solution. All the reactions in Entries 1-9 were conducted by alternately titrating 0.25 mol of palmitoyl chloride in 50 ml acetone and 0.1 N sodium hydroxide solution within the assigned pH range. The reactions were maintained at 15°C for 4 hr and allowed to stand at room temperature overnight. The resulting reaction mixtures were then acidified with 1 N HCl solution. The percentage yields were recorded on the basis of the amount of dried precipitate obtained after acidification of the reaction mixture and removal of the liquid phases by vacuum filtration relative to the total amount of starting reagent used.

Method for Determination of Percentage of Component in Crude Product Mixture

The percentage of component detected in the product mixtures were assayed based on the percentage area under the peak of the GC-FID chromatogram carried out. The GC-FID was performed using Hewlett-Packard 5890-II gas chromatography equipped with Automatic Liquid Sample (ALS) injector and a flame ionisation detector (detector temperature 380°C). Zebron-5 HT Inferno capillary column (30 m × 0.25 mm i.d., Phenomenex, USA) was specially coated with 0.25 μm of 5% phenyl – 95% dimethylpolysiloxane. Helium was used as the carrier gas. The silylated di-palmitoyl hydroxyproline standard was dissolved in 1 ml of dimethylformamide and 1 ml of dicholormethane. The 1 μl of the prepared standard was injected into GC at 120°C (1 min) then programmed up to 350°C (4 min) at 6°C min^{-1}. The percentage composition of component was represented by the percentage area under the peak of the chromatogram.

RESULTS AND DISCUSSION

Generally, L-hydroxyproline is an amino acid with 2 nucleophiles on the same molecule, a pyrrolidine amino group and a pyrrolidin-3-ol hydroxyl group. The pKa value of the pyrrolidine amino group is 10.73 (Burton *et al*., 1959). On the basis of our study, in an aqueous acetone medium with an assigned pH range of 9.0-10.0, acylation of L-hydroxyproline only occurred at the pyrrolidin-3-ol hydroxyl group, where the palmitic acid was identified as the major by-product. *Table 1* summarises the percentage yield of product mixture and percentage composition, reported as the percentage of area under peak for each of the assigned pH range of reaction medium denoted as Entries 1-9.

The percentage yield of the product mixtures, isolated after the acidification varies significantly even though the same amount of starting material were used during the acylation reactions. At this point of the study, the variations in percentage yield were expected to be due to the losses encountered during neutralisation with NaOH solution, acidification with HCl and washing step. Incomplete acidification may result in losses of palmitic acid, in the form of sodium palmitate. Since sodium palmitate dissolved in the aqueous medium, it will then be drained away as filtrate during the filtration step.

On the basis of product composition of the reactions conducted according to Entries 1-5 and assayed by GC-FID, *O*-palmitoyl hydroxyproline and palmitic acid were detected as the main product and by-product, respectively. The pH range of 9.0-10.0 assigned for the reaction medium is therefore not high enough to enable the pyrrolidine amino group to be acylated by palmitoyl chloride. Thus, only *O*-palmitoyl hydroxyproline and palmitic acid were detected in the product mixture of Entries 1-5.

When the assigned pH range was increased to 11.0 and 12.0, conducted according to Entries 6 and 7, respectively, there was a drop in percentage composition of *O*-palmitoyl hydroxyproline. The drop in the percentage composition of this product was then observed to be replaced by the formation of new peak on the GC-FID, which was then confirmed by GC-MS, to be that of the *N,O*-di-palmitoyl hydroxyproline. From this observation, it was deduced that *N,O*-di-palmitoyl hydroxyproline occurs when the pH is maintained near to the pKa value of hydroxyproline's amino group. The adjusted pH condition of the solution would, subsequently, enable the formation of an amide linkage between the *O*-palmitoyl hydroxyproline and palmitoyl chloride and not through simultaneous acylation of both the pyrrolidine amino and pyrrolidine-3-ol groups of the L-hydroxyproline. On the basis of the product composition Entries 8 and 9, the percentages of *N,O*-

TABLE 1. EFFECT OF ASSIGNED pH RANGE OF AQUEOUS ACETONE MEDIUM ON THE YIELD, COMPOSITION OF PRODUCTS FROM ACYLATION OF L-HYDROXYPROLINE AND PALMITOYL CHLORIDE

Entry	pH range[a]	Yield (%)	Percentage of *O*-palmitoyl hydroxyproline (%)	Percentage of *N,O*-di-palmitoyl hydroxyproline (%)	Percentage of palmitic acid (%)	Others[b]
1	9.0-10.0	87.8	36.07	-	55.03	8.9
2	9.0-10.0	89.9	47.11	-	48.43	4.46
3	9.5-10.0	67.2	46.96	-	43.91	9.13
4	9.0-9.5	89.3	45.05	-	49.51	5.44
5	9.0-10.0	78.5	40.68	-	52.19	7.13
6	10.5-11.0	73.0	35.17	4.79	50.42	9.62
7	11.5-12.0	97.0	22.66	10.78	49.86	16.7
8	12.0-13.0	81.3	27.9	10.34	50.98	10.78
9	13.0-13.3	58.7	16.25	9.00	57.06	17.69

Note: [a]The range of pH studied by alternating the titration of palmitoyl chloride and 0.1 N sodium hydroxide solution. [b]Total percentage of other components as detected on GC-FID chromatogram. The characterisation of the other components was not reported in this study.

di-palmitoyl hydroxyproline assayed decreased when the assigned pH range of the reaction medium was increased to more than 13.0. These results were attributed to the pH of the medium being too high, which resulted in hydrolysis of the *N,O*-di-palmitoyl hydroxyproline and *O*-palmitoyl hydroxyproline into L-hydroxyproline and palmitic acid. The L-hydroxyproline was then washed away by the aqueous filtrate and would not be detected in the yield recovered. This observation is in accordance with the very low percentage yield of only 59%, obtained from the reaction conducted according to Entry 9.

Therefore, on the basis of the various assigned pH range of the reaction medium, the following schematic reaction pathways of the acylation reaction between L-hydroxyproline (I) and palmitoyl chloride in aqueous acetone medium, resulted in products denoted as II-VIII, was suggested as illustrated in *Figure 1*.

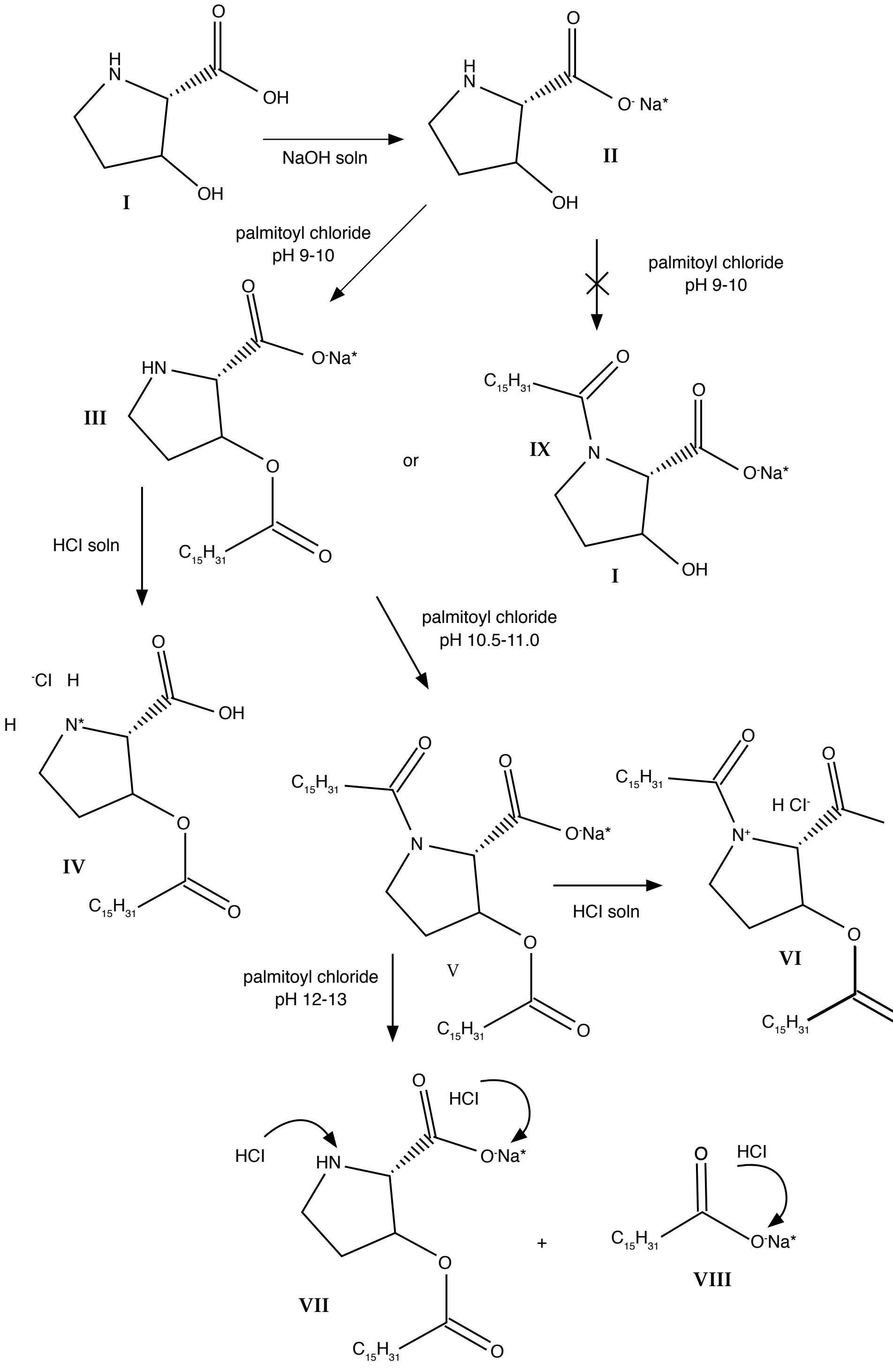

Figure 1. Possible schematic reaction pathways for the acylation of L-hydroxyproline (I) and palmitoyl chloride in aqueous acetone medium of varying pH range giving products II-VIII.

CONCLUSION

The pH of an acylation reaction medium carried out through the Schotten-Bauman conditions is one of the most important parameters in ensuring the maximum performance of the acylation reaction to get to target product. For acylation of the 2 nucleophiles, of different electronegativity, on L-hydroxyproline, the pH of the medium needs to be adjusted to the pKa value of the nucleophiles to be acylated. In this study, it was concluded that acylation at the pH range of 9-10, predominantly formed *O*-palmitoyl hydroxyproline and palmitic acid. The formation of *N,O*-di-palmitoyl hydroxyproline would only be detected within the range of 4.79% to 10.78% when the pH range was regulated within 10.5-12.0. However, in the attempt to increase the percentage of the *N,O*-di-palmitoyl hydroxyproline by increasing the pH of the reaction medium to above 12.0 resulted in hydrolysis of the palmitoyl chloride, *O*-palmitoyl hydroxyproline and *N,O*-di-palmitoyl hydroxyproline giving back the L-hydroxyproline and palmitic acid. This finding is important in determining the right strategy to conduct acylation of such a pH dependence reaction.

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