박 성 순 교수 지도

석사학위 청구논문

Facile synthesis and application of nano-sized hydroxyapatite for immobilization of subtilisin

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이윤재의 석사학위 논문으로 인준함

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논 문 개 요

하이드록시아파타이트(hydroxyapatite)는 뼈의 주된 구성 성분으로서 환경친화적이고 생체친화적이기 때문에, 많은 생명공학적인 응용에 활용되고 연구되어 왔다. 이러한 하이드록시아파타이트의 응용연구와 함께 하이드록시아파타이트의 제조법에 관한 연구 역시 연구자들의 주된 관심사이다. 그렇지만, 하이드록시아파타이트의 합성에 있어서 대부분의 경우 오랜 시간이 요구되는 하소과정(煆燒, calcination)이 포함된다. 본 연구에서는 이러한 하소과정이 생략된 보다 간편하고 빠른 시간 안에 하이드록시아파타이트를 제조하는 방법을 개발하였고, 하이드록시아파타이트를 활용하여 단백질 가수분해 효소인 subtilisin의 고정화를 통해 유기용매 하의 반응성을 향상시켰다.

먼저, 하이드록시아파타이트는 하소과정과 특별한 주형(template)화합물 없이, 마이크로웨이브의 조사를 통해 짧은 시간 안에 합성되었다. 합성된 하이드록시아파타이트는 SEM 분석에 의해 나노크기의 막대모양을 지니고 있음이 확인되었다. 그리고 XRD, IR 분석에 의해 기존에 알려진 하이드록시아파타이트의 구조와 동일한 것으로 확인되었다. 하이드록시아파타이트의 생명공학적 응용성을 확대하기 위해서, 하이드록시아파타이트 합성 시에 아미노산을 첨가한 결과, 기존 하소과정이 포함된 합성방법으로는 생성하기 불가능했던 아미노산이 표면에 결합된 하이드록시아파타이트를 얻을 수 있었다. 결합된 아미노산의 존재는 강산으로 분해된 아미노산-하이드록시아파타이트의 시료를 NMR 분석을 통해 확인하였다.

리파제와 함께, 광화학적으로 순수한 이차 알코올을 합성하는데 활용되는

subtilisin은 리파제와 달리 유기용매 하에서 그 반응성이 현저히 감소된다. 따라서 연구자들은 subtilisin의 유기용매 하에서의 반응성을 향상시키기 위해 시도하여 왔다. 본 연구에서는 subtilisin의 표면이 극성임에 착안하여, 극성표면을 지닌 하이드록시아파타이트를 고정화용 지지체로 사용하면, 이들 간의 긍정적인 상호작용을 할 수 있을 것으로 기대하였고, 결과적으로 subtilisin의 유기용매 하에서 반응성이 향상될 것으로 기대하였다. 기대와 마찬가지로, 하이드록시아파타이트와 함께 동결건조된 subtilisin은 1-phenylethanol과 vinyl butyrate의 반응에서 1000배 이상의 반응성 향상을 보여주었다. 이 결과는 하이드록시아파타이트가 subtilisin의 고정화 지지체로 효과적으로 활용될 수 있음을 보여준다.

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Chapter 1. Introduction

1.1 Hydroxyapatite

Hydroxyapatite (HAp) is a natural bone material. Formula of HAp is $Ca_{10}(PO_4)_6(OH)_2$, and the shape is varied depending on the synthetic method, the pH of a synthetic solution, and the calcination temperature.[1] Especially, nanocomposites of hydroxyapatite has advantages of larger surface area, higher surface reactivity, relatively stronger interfacial-binding, more design flexibility, more enhanced mechanical reliability than monolithics and microcomposites. The nanocomposites have been used in orthopaedics, tissue engineering, and drug delivery.[2]

1.1.1 Synthesis of hydroxyapatite

Researchers have developed the synthetic methods of hydroxyapatite. There are four major methods: dry methods, wet methods, alternative energy input methods, and other methods (Fig 1). Although dry methods provide properly crystallized hydroxyapatite, there is a drawback that the synthesis must be carried at high temperatures (1050 °C). Wet method is relatively a convenient method to be conducted, and generally produces hydroxyapatite with high yields. However, the well-defined control of solvent, pH, and temperature is essential to prepare the crystalline structure. Alternative energy input methods also provide good crystal

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1) Sadat-Shojai, M., Khorasani, M. T., Dinpanah-Khoshdargi, E., & Jamshidi, A. 2013. *Acta biomaterialia*, 9(8), 7591-7621.

2) Murugan, R., & Ranakrishna, S. 2005. *Composites Science and Technology*, 65(15-16), 2385 - 2406.

structure and can make nano-sized porous hydroxyapatite using template compounds, but it requires an extra template compound and careful washing steps.[3)]

In this thesis, microwave-irradiation method, one of the alternative energy input methods, was used to synthesize nano-sized hydroxyapatite.

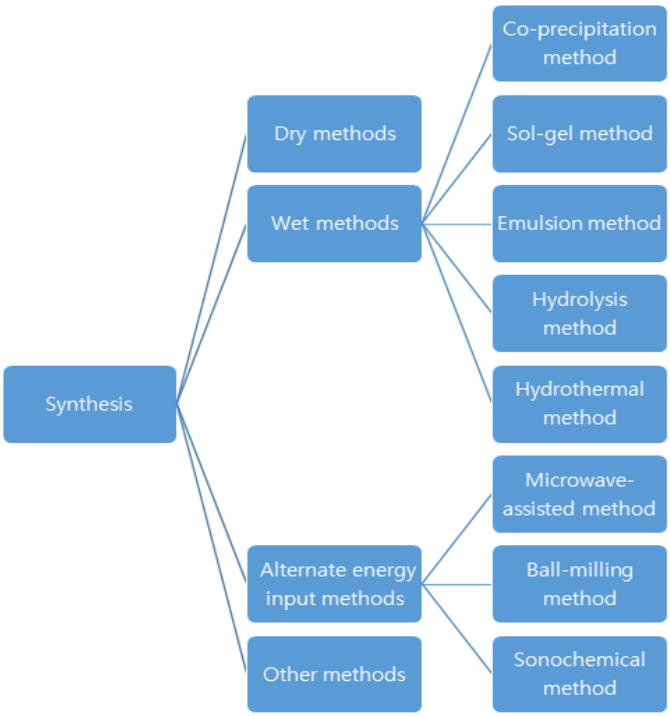

Figure 1. Synthesis methods of hydroxyapatite.[4)]

3) Thirumalai, J. 2018. *Biomedical Applications and Its Technological Facets*, 1. 4
4) Fihri, A., Len, C., Varma, R. S., & Solhy, A. 2017. *Coordination Chemistry Reviews*, 347, 48-76.

1.2 Protease from *Bacillus licheniformis* (subtilisin)

Subtilisin is a member of serine proteases. It has 269 residues with Ca^{2+} cofactor, and has molecular weight of 27 kDa.[5] The catalytic triad of subtilisin is composed of Asp-His-Ser,[6] which is similar to that of lipases. Subtilisin has an α/β protein scaffold (Figure 2).[7] Subtilisin has high activity in water contents and has enantioselectivity toward chiral *sec*-alcohols (*S*-selectivity).[8]

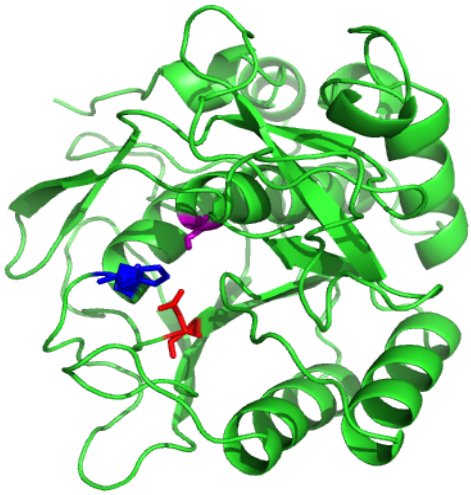

Figure 2. A crystal structure of Subtilisin Carlsberg (PBD code: 1AF4). The catalytic triad (Asp 32, His 64, and Ser 221) is shown in a stick model (red, blue and pink, respectively).

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5) Syed, R., Wu, Z. P., Hogle, J. M., & Hilvert, D. 1993. *Biochemistry*, 32(24), 6157-6164.
6) Polaina, J., & MacCabe, A. P. 2007. I*ndustrial enzymes*, 200-209. Springer.
7) Siezen, R. J., & Leunissen, J. A. 1997. *Protein science*, 6(3), 501-523.
8) Kim, M. J., Chung, Y. I., Choi, Y. K., Lee, H. K., Kim, D., & Park, J. 2003. *Am. Chem. Soc*, 125(38), 11494-11495.

1.3 Immobilization

There are several reasons for immobilizing enzymes. For example, it is needed for higher operational times of enzyme and for increasing their total turnover numbers. Besides, enzymes often has to be used in non-conventional media to increase the concentrations of substrates for intensification of processes or to shift the reaction equilibrium, to perform a solvent-free reaction system, and to prevent from microbial contaminations.[9)]

1.4 Outline of this thesis

Hydroxyapatite (HAp) has similarity in the crystallinity and chemical composition to that of human hard tissue so that it is suitable for substitution of bone materials.[10) 11)] Researchers have developed the various synthetic methods of hydroxyapatite. Among them, the microwave-irradiation method can be easily used, and provide well-crystallized hydroxyapatite with micro-sized porous structures. This thesis deals with a synthetic method of hydroxyapatite by microwave-irradiation. The method developed by the current investigation provides well-crystallized nano-sized hydroxyapatite for a short period of time. In addition, it has been studied that nano-sized hydroxyapatite can be used as a supporting material for the immobilization of subtilisin to improve the activity in organic media.

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9) Jose M. Guisan, Juan M. Bolivar, Fernando López-Gallego, Javier Rocha-Martín 2006. *Immobilization of enzymes and cells*, 22,1-4.

10) Kantharia, N., Naik, S., Apte, S., Kheur, M., Kheur, S., & Kale, B. 2014. *Bone*, 34(15.2), 1-71.

11) Fihri, A., Len, C., Varma, R. S., & Solhy, A. 2017. *Coordination Chemistry Reviews*, 347, 48-76.

Chapter 2. Microwave-assisted facile synthesis of amino-acid decorated hydroxyapatite

Abstract

Hydroxyapatite is a main component of the natural bone system, and has been garnered much attention in nano-medical applications. For such applications, researchers have developed the synthetic methods of hydroxyapatite with various sizes and morphologies. Besides, interest in the surface modification of the hydroxyapatite has also increased recently because the modified surface can improve interaction with biomolecules, such as proteins. Most synthetic procedures contain thermal treatment process (*i.e.* calcination) for proper crystallization or removal of template molecules used after hydroxyapatite formation. The thermal treatment process generally requires a long period of time (typically 10 h) and large energy consumption (typical temperature: 500 ℃).[12] Hence, development of a facile synthetic process of nano-sized hydroxyapatite is important in the application of hydroxyapatite. Microwave-assisted synthesis would be an alternative process to shorten the synthesis time because microwave irradiation can introduce energy for a short period of time. In this study, a microwave - irradiation method was employed to synthesis of hydroxyapatite. Microwave irradiation efficiently produced nano-sized hydroxyapatite for 90 min without any template molecules. In addition, amino-acid-decorated hydroxyapatite was also prepared by addition of

12) Amer, W., Abdelouahdi, K., Ramananarivo, H. R., Zahouily, M., Fihri, A., Djessas, K., Zahouily,K., Varma, R.S., & Solhy, A. (2014). *CrystEngComm*, 16(4), 543-549.

amino acids during microwave-assisted syntheses. As a result, all seventeen natural amino-acid- (except glutamine, cystine, and arginine) and four unnatural amino-acid-decorated hydroxyapatites were synthesized. The prepared hydroxyapatite was characterized by SEM, XRD, IR, and NMR.

2.1 Introduction

Hydroxyapatite (HAp) is one of the main components in natural bone systems and is environmentally friendly and bio-comparable materials. Researchers have employed HAp to many bio-applications and also developed its synthetic methods (for example, dry methods, wet methods, alternate energy input methods, other methods, etc.)[13] A few reaction factors in the HAp synthesis were revealed to be critical for controlling the morphology of HAp. For instance, pH, template molecules, and temperature of calcination influence the shape and size of HAp.[14] Besides, most methods require a calcination procedure to crystallize HAp and to remove the template molecules used. Such a calcination procedure requires a long period of time and large energy consumption, which do not coincide with environmental concerns. Hence, the development of a facile method has gained more attention. Microwave irradiation can be an alternative method because it is suitable in applying energy to a reaction system for a short period of time.[15] In this study, microwave-assisted synthesis of

13) Fihri, A., Len, C., Varma, R. S., & Solhy, A. 2017. *Coordination Chemistry Reviews*, 347, 48-76.
14) Sadat-Shojai, M., Khorasani, M. T., Dinpanah-Khoshdargi, E., & Jamshidi, A. 2013. *Acta biomaterialia*, 9, 7591-7621.
15) Farzadi, A., Solati-Hashjin, M., Bakhshi, F., & Aminian, A. 2011. *Ceramics*

HAp has been investigated.

2.2 Result and discussion

Screening the reaction time and pH of the reaction mixture

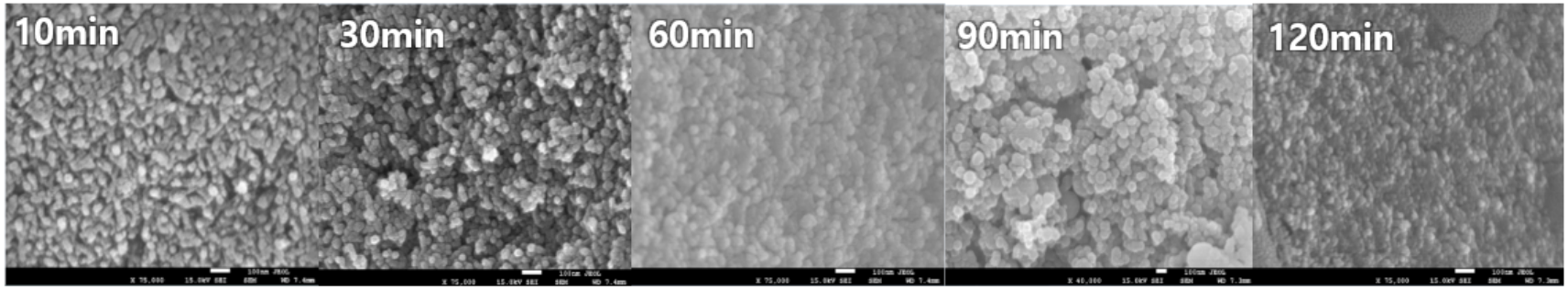
After fixing the molar ratio of calcium ion and phosphate ion to 1.67, template-free hydroxyapatite was prepared by using microwave irradiatio n.[16] To optimize the reaction condition, the time of microwave irradiation and pH of an $(NH_4)_2HPO_4$ solution were screened (Figures 1 and 2). First, the irradiation time (reaction time) was varied as 10, 30, 60, 90, and 120 min at pH 10.8. The SEM analyses suggested that microwave irradiation is required at least for 90 min to observe clear-shaped particles (Figure 1(a)), although the XRD patterns are all similar (Figure 1(b)). However, the XRD patterns were distinct from those of the commercial HAp. Hence, another factor, pH in the current system, for synthesis of hydroxyapatite has to be investigated. The pH of the reaction solution was varied from 10.2 to 10.8, and the hydroxyapatite obtained was analyzed by SEM and XRD. While the SEM images are all similar, the XRD patterns at pH 10.6 are clearly distinct from the others and identical to those of the commercial hydroxyapatite (Figure 1(b)). The shape of synthesized HAp was shown as rod type. The size of the synthesized HAp is 80-150 nm in height and 20-25 nm in width. Besides, the peaks of IR for HAp synthesized at pH

International, 37, 65-71.

16) Fihri, A., Len, C., Varma, R. S., & Solhy, A. 2017. *Coordination Chemistry Reviews*, 347, 48-76.

10.6 are also identical to those of the commercial HAp (Figure 3).

(a)



(b)

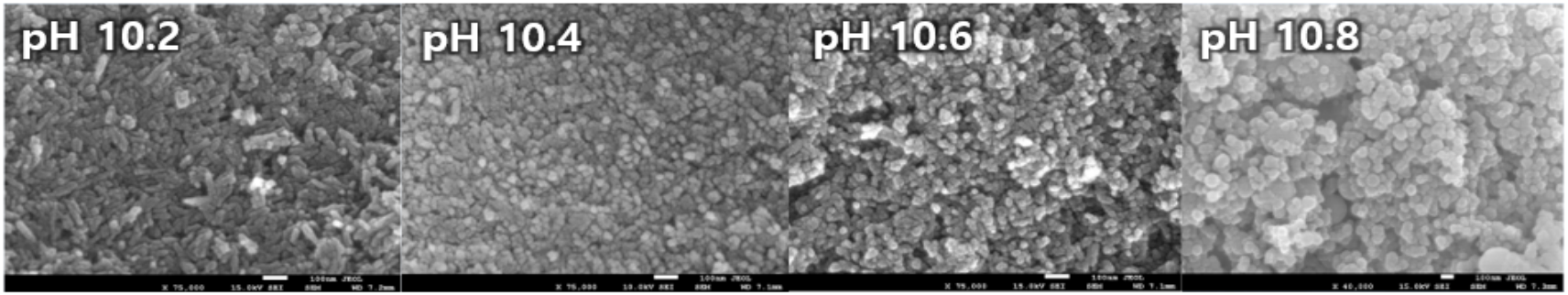


Figure 1. SEM images of HAps from different reaction conditions. a) Irradiation time varied at pH 10.8. (b) pH of the reaction solution varied. The scale bar = 100 nm.

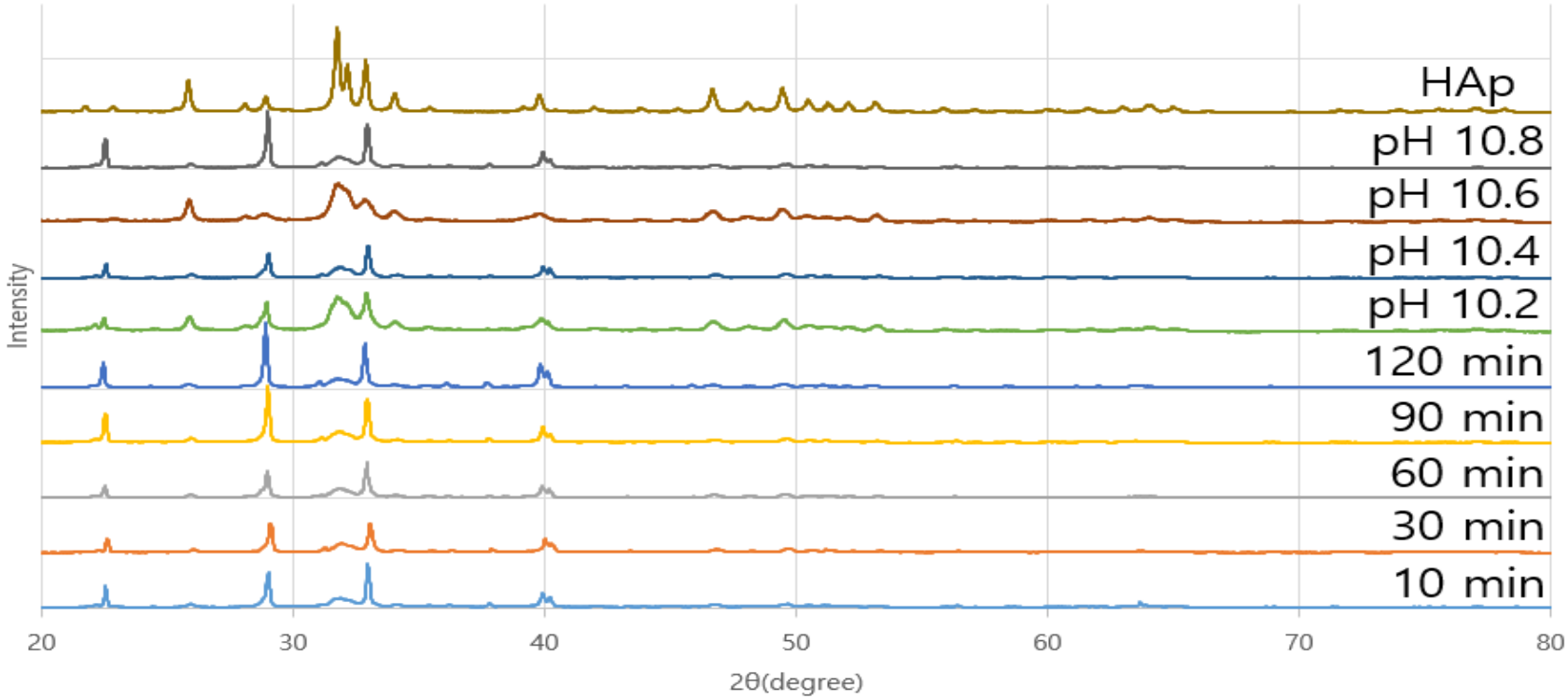


Figure 2. XRD analyeses of the commercial and synthesized HAps.

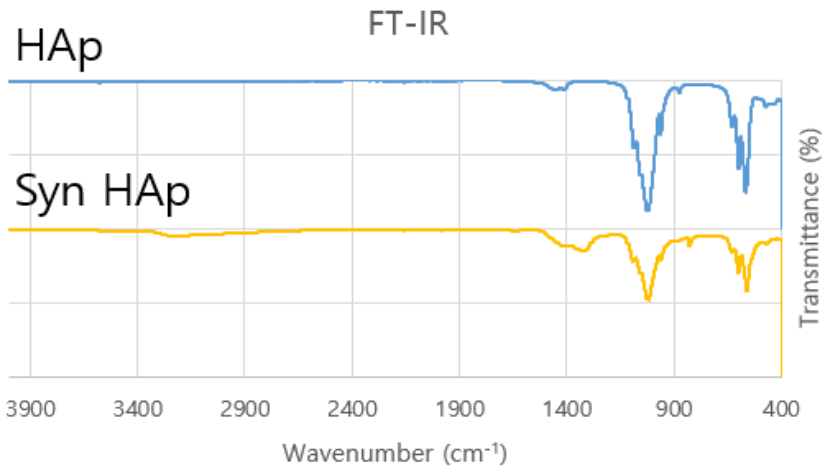


Figure 3. IR analyses of the commerical and synthesized HAp.

Synthesis of amino-acid-decorated HAp

Amino acids coated on hydroxyapatite may provide more specific interaction with proteins because the side chains of amino acids are the actual channel for protein-protein interaction. Hence, the currently developed method was employed to prepare amino-acid-decorated hydroxyapatite. As a representative, synthesis of glycine-coated hydroxyapatite was attempted by three different process, and the amount of glycine on hydroxyapatite were compared. In the method 1, glycine was added in the reaction mixture containing all components, and microwave irradiation was applied to the reaction mixture. In the method 2 and 3, the synthesized hydroxyapatite was, first, suspended in ammonium hydroxide solution (0.01 M, pH 10.6) and then glycine was dissolved in the solution. In the method 2, microwave irradiation was then employed, but not in the method 3. The glycine-decorated hydroxyapatites were digested with a DCl solution (5% in D_2O), and the amount of glycine was estimated using

t-butanol as a internal standard by NMR. Among the methods, the method 1 provide the highest amount of glycine (Table 1). Hence, the method 1 was employed to synthesize other amino-acid-decorated hydroxyapatite. As a result, sixteen natural amino-acid (except glutamine, cystine, and arginine) and three unnatural amino-acid decorated hydroxyapatite was synthesized.

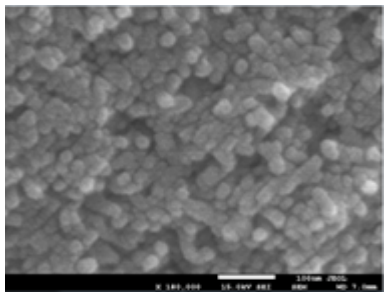
Table 1. H^1-NMR of Amino acid contents of HAp- amino acid. H^1-NMR of synthesized HAp with amino acid, HAp with amino acid using or not using microwave. The rate of amino acids were calculated by using tert-butanol.

	Amino acid	Contents (per 0.1 g, g)
Method 1	glycine	4.78×10^{-4}
	proline	2.26×10^{-4}
	valine	5.90×10^{-4}
	leucine	1.38×10^{-4}
	isoluecine	1.43×10^{-4}
	methionine	4.17×10^{-4}
	phenylalanine	4.57×10^{-4}
	tryptophan	6.77×10^{-4}
	serine	5.48×10^{-4}
	threonine	3.81×10^{-4}
	asparagine	2.65×10^{-4}
	aspartic acid	5.74×10^{-4}
	glutamic acid	5.63×10^{-4}
	lysine	5.65×10^{-4}
	histidine	4.07×10^{-4}
	tyrosine	$4.58 *10^{-4}$
	β-alanine	7.04×10^{-4}
	ɣ-aminobutyric acid	7.11×10^{-4}
	6-aminocaproic acid	$5.62 *10^{-4}$
Method 2	glycine	3.71×10^{-4}
Method 3	glycine	1.88×10^{-4}

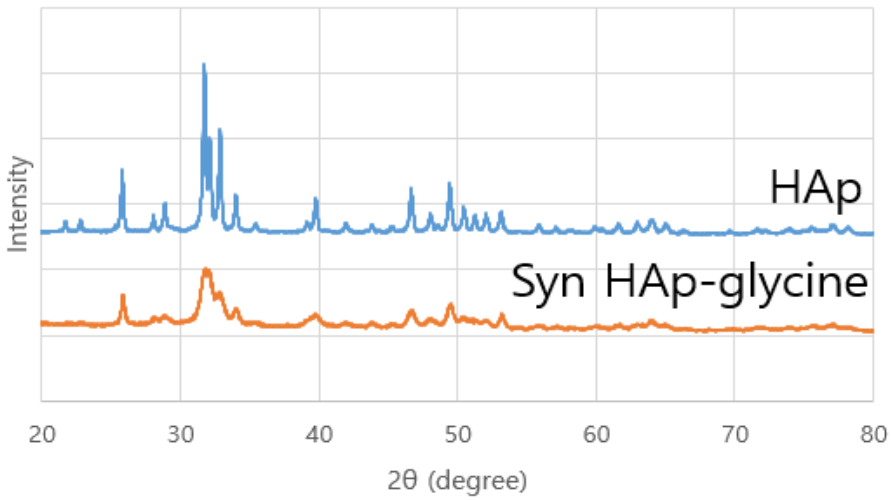
SEM, XRD, and IR ananlyses of glycine-decorated hydroxyapatite

SEM analyses exhibited that the morphology of the glycine-decorated hydroxyapatite was identical to the hydroxyapatite synthesized by microwave irradiation. The synthesized HAp with glycine were shown as rod type (Figure 4(a)). The XRD and IR patterns were also identical to those of the commercial hydroxyapatite (Figures 4(b) and (c)). Analyses for the other amino-acid-decorated HAp also exhibited the similar results (see Appendix).

(a)



(b)



(c)

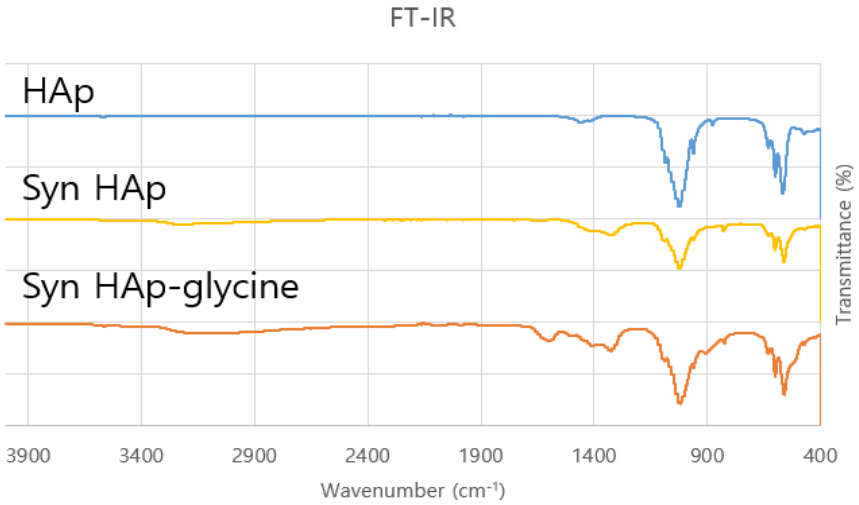


Figure 4. SEM (a), XRD (b) and IR (c) of HAp and synthesized HAp-glycine. The scale bar of SEM = 100 nm.

2.3 Experiment section

General methods

Syntheses of hydroxyapatite or amino - acid decorated hydroxyapatite using microwave irradiation

In a 100 mL bottle, 0.05 mol of $Ca(NO_3)_2 \cdot 4H_2O$, and 0.02 mol of amino acid were mixed in 75 mL of deionized water.

In another 500 mL bottle, 0.03 mol of $(NH_4)_2HPO_4$ was dissolved in 125 mL of deionized water and set the pH (10.2, 10.4, 10.6, or 10.8) by adding NH_4OH.

Two of them were mixed. 25 mL of the mixture was added into 35 mL

microwave tube. Microwave tube was added into microwave and set the time (10, 30, 60, 90 or 120 min) in 80 ℃, and 50 W. Repeated 4 times. After using microwave, product was washed with 20% EtOH and deionized water for three times.

After washing, it was dried into 80 ℃ oven for 24h.

Binding hydroxyapatite with amino acid using microwave

100mg of HAp and amino acid were added and mixed with water (pH 10.6, NH_4OH). Microwave tube was replaced into microwave and set the time in 90 min. Set the temperature in 80 ℃, pressure in 17 bar and power in 50 W. After using microwave, centrifuge for 10 min and rid off the supernatant. The product was washed with 20% EtOH and deionized water for three times. After washing, put it into 80 ℃ oven for 24 h.

Binding hydroxyapatite with amino acid not using microwave

100mg of HAp and amino acid were added and mixed with water (pH 10.6, NH_4OH). Product has shaken for overnight in room temperature. Centrifuge for 10 min and rid off the supernatant. The product was washed with 20% EtOH and deionized water for three times. After washing, the product was dried at room temperature.

Amino acid content calculation method using NMR

In 1.5 mL ep tube, 100 mg of sample and 5 μL of tert-Butanol was added into 995 μL of $DCl{\cdot}D_2O$ (5% w/w). Peaks of sample and t-Butanol were calculated in ratio.

General peak of IR (hydroxyapatite)

In commercial hydroxyapatite (HAp), peak of PO_4^{3-} (473.22 cm^{-1}, 961.8 cm^{-1}, 1024.21 cm^{-1}, 1086.00 cm^{-1}), peak of HPO_4^{3-} (565.87 cm^{-1}, 599.76 cm^{-1}), peak of CO_3^{2-} (1412.94 cm^{-1}, 1456.58 cm^{-1}), and peak of OH^- (3572.54 cm^{-1}) were shown. In synthesized HAp, peak of PO_4^{3-} (470.91 cm^{-1}, 961.60 cm^{-1}, 1022.84 cm^{-1}, 1088.87 cm^{-1}), peak of HPO_4^{3-} (560.77 cm^{-1}, 600.71 cm^{-1}), peak of CO_3^{2-} (1324.27 cm^{-1}, 1419.02 cm^{-1}), peak of H_2O (3222.95 cm^{-1}) and peak of OH^- (3568.49 cm^{-1}) were shown.

Chapter 3. Hydroxyapatite-assisted enhancement of subtilisin activity in organic solvents

Abstract

Subtilisin is one of the useful enzymes in kinetic resolution of chiral *sec*-alcohol. However, the activity of subtilisin significantly decreases in organic media. Therefore, researchers have developed to improve its activity in organic media. Many supporting materials for enzyme immobilization is porous hydrophobic materials. However, the surface of subtilisin is rather polar, and thus hydrophobic materials are not suitable for immobilization of subtilisin. Instead, nano-sized hydroxyapatite can a candidate as supporting materials because hydroxyapatite is environmentally benign and possesses highly polar surface. It can be hypothesized that highly polar surfaces of hydroxyapatite can build positive interactions with the surface of subtilisin, and thus preventing subtilisn from aggregation. The hypothesis was exploited in this study. Subtilisin was immobilized by lyophilization with hydroxyapatite. The contents of hydroxyapatite were varied as 80%, 90%, 95%, and 98%. The activity of subtilisin was measured in transesterification of 1-phenylethanol with vinyl butyrate in organic solvents. As expected, the activity of subtilisin was dramatically improved by a factor of 1000. This result exhibits that hydroxyapatite is suitable materials for immobilization of subtilisin.

3.1 Introduction

Kinetic resolution is one of the most useful methods to prepare enantio-pure chiral *sec*-alcohols. For this, lipase is most commonly used because lipase possesses high enantioselectivity, thermostability, and chemo-tolerance. In addition, subtilisin (protease from *Bacillus licheniformis*) is often used for kinetic resolution of chiral *sec*-alcohols because subtilisin possesses the opposite enantioselectivity (*i.e.* *S*-selectivity). The environment of the active site in subtilisin is a mirror image to that of lipase.[17] However, subtilisin exhibits a significant decrease of the activity in organic solvents compared to that in a aqueous system. [18]

Hence, enhancing the activity of subtilisin in organic solvents has been tried by many research groups. For example, Dordick *et al.* reported that lyophilization with KCl (98%) enhanced the activity of subtilisin in organic solvents by a factor of 3000.[19] Kim *et al.* utilized iron oxide nanoparticles to enhance the activity of subtilisin in organic solvents.[20] The common feature of the materials is that their surface is highly charged. This charged surface can positively interact with the polar surface of subtilisin, and thus subtilisin can be stabilized. One of the environmentally friendly materials possessing the charged surface is hydroxyapatite. Hydroxyapatite is easy to be handled than the KCl-subtilisin system and is more

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17) Cha, H., Park, J. & Park, S. *Biotechnol Bioproc E* **24**, 41 - 47

18) Klibanov, A. *Nature* **2001**, 409, 241 - 246.

19) Yuri L. Khmelnitsky, Stephanie H. Welch, Douglas S. Clark, and Jonathan S. Dordick *J. Am. Chem. Soc,* **1994,** 116, 2647-2648

20) Cheng-gang Ci, Hong-bo Yu, Su-qin Wan, Jing-yao Liu, Chia-Chung Sun. *Bull. Korean Chem. Soc.* **2011**, 32, 2871-2872

bio-degradable than iron-oxide. Hence, hydroxyapatite may be an alternative supporting material to stabilize subtilisin in organic solvents. In this study, we investigated the activity of subtilisin lyophilized with hydroxyapatite in organic solvents.

3.2 Result and discussion

Immobilization of subtilisin on hydroxyapatite (HAp)

Our research group previously reported that utilizing a linker compound (6-aminocaproic acid) is necessary to covalently bind lipase, possessing the hydrophobic surface, to hydroxyapatite (HAp).[21] Unlike lipase, protease from *Bacillus licheniformis* (subtilisin) possesses more charged amino acids on its surface. This implies that a charged nano-sized material, such as hydroxyapatite, can efficiently bind with subtilisin, and thus the activity of subtilisin in organic solvents can be enhanced. In fact, lyophilization with KCl (98%) improved the activity of subtilisin in organic solvents by a factor of about 3000. The immobilization was simply carried out by lyophilization with a particular amount of hydroxyapatite. For comparison, lyophilization with KCl (98%) was also conducted.

SEM, XRD, and IR analyses of HAp and HAp-subtilisin

The morphology change was monitored by a analyses of SEM (scanning electron microscope). The SEM image of hydroxyapatite and HAp-subilisin exhibited that the morphology of HAp was not altered during lyophilization (Figure 1).

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21) Minjeong Jeon, Suhyun Jung and Seongsoon Park *New J. Chem.*, **2018**, 42, 14870-14875

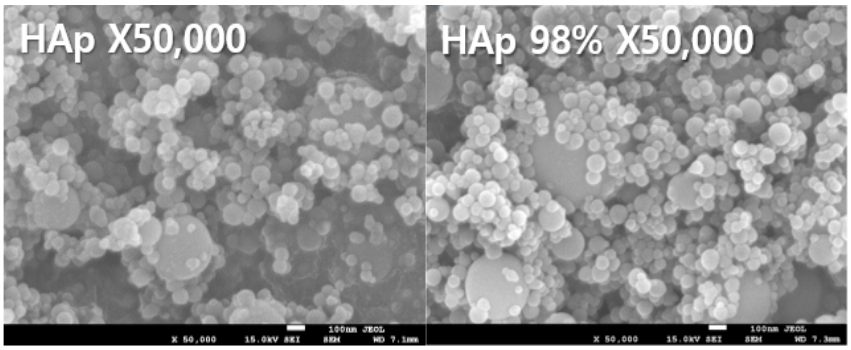
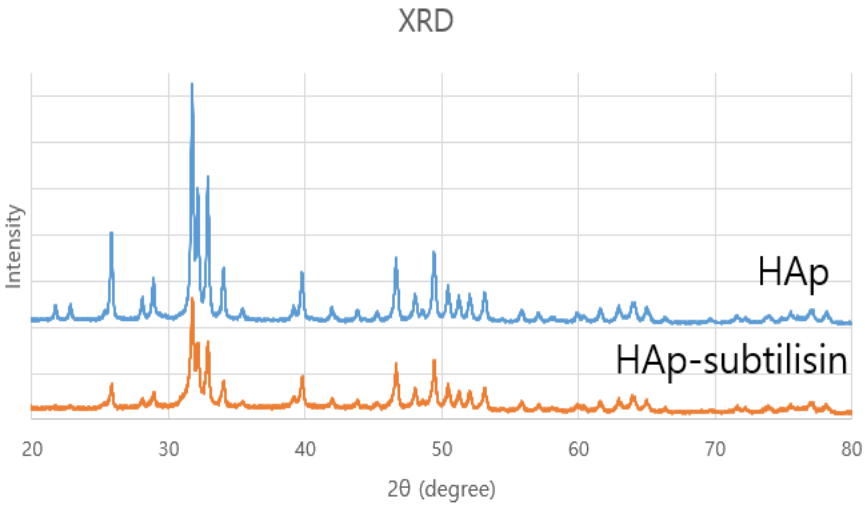


Figure 1. SEM analyses of HAp and HAp-subtilisin. The scale bar = 100 nm.

XRD and IR of HAp-subtilisin exhibited same pattern as HAp (Figure 2).



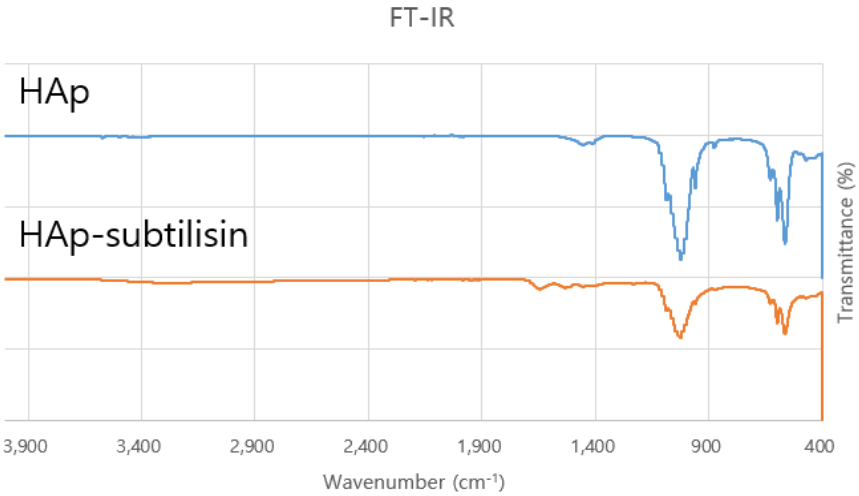


Figure 2. XRD and FT-IR of HAp and HAp-subtilisin.

Comparison of the effects of KCl and HAp on the activity of subtilisin in various organic solvents

Five solvents were screened through transesterification (methyl tert-butyl ether (MTBE), cyclohexane, diisopropyl ether, n-hexane, tetrahydrofuran (THF)). For transesterification, (±)-1-phenylethanol and vinyl butyrate were used. The data of 98% of KCl and 98% of HAp in 24 h were detected by Gas Chromatography. The result (Figure 3) shows that conversion of 98% of HAp is higher than 98% of KCl in 5 solvents. Especially, 98% of HAp is three times higher than 98% of KCl in MTBE.

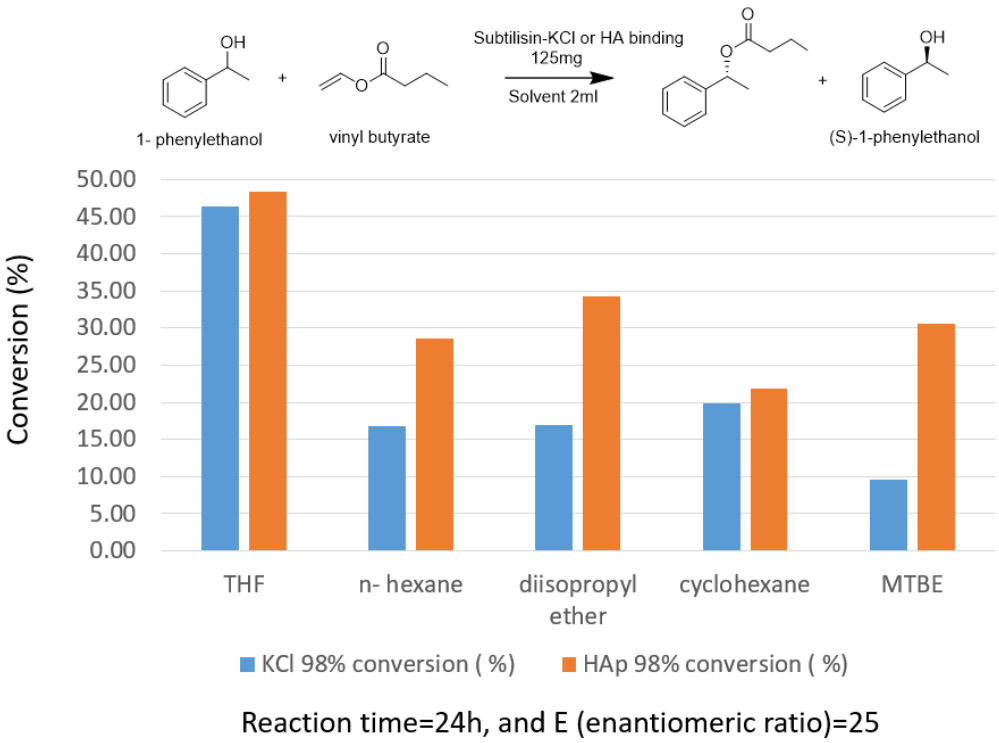


Figure 3. Solvent screening of KCl 98% and HAp 98% (24 h). Using (±)-1-phenylethanol (1 mmol) and vinyl butyrate (4 mmol) as substrate, lyophilized subtilisin of *Bacillus licheniformis* with KCl or HAp (125 mg) as catalyst, molecular seives (25 mg) to control water contain and 5 solvents (2 mL) were screened. For comparison, 24h was examined. E=25.

Through 5 solvents screening, tetrahydrofuran (THF) has highest conversion rate. So it was chosen to test specific activity of HAp-subtilisin binding.

Measurement of specific activity

Subtilisin contents were tested 2% to 20%. The data (Table 2) showed higher specific activity in order of 98% of HAp, 95% of HAp, 90% of HAp , and 80% of HAp. 98% of synthesized HAp showed five times lower than

98% of HAp.

Table 1. Specific activity and enantiomeric ratio of HAp-subtilisin binding.

entry	enzyme	specific activity ($mmol \cdot min^{-1} \cdot mg^{-1}$)
1	subtilisin	n.d
2	HAp 98%- subtilisin	6.13×10^{-4}
3	HAp 95%- subtilisin	2.64×10^{-4}
4	HAp 90%- subtilisin	1.65×10^{-4}
5	HAp 80%- subtilisin	0.92×10^{-4}
6	mn-HAp 98%- subtilisin	1.47×10^{-4}

Subtilisin E= n.d., E=25.

Recycling of KCl 98% and HAp 98%

Recycling experiments were conducted seven times (Figure 4). The initial conversion value (24 h) was calculated with 100%. After 7^{th} recycling, conversion rate of KCl (98%) was decreased 90% and conversion rate of HAp (98%) was decreased 70%.

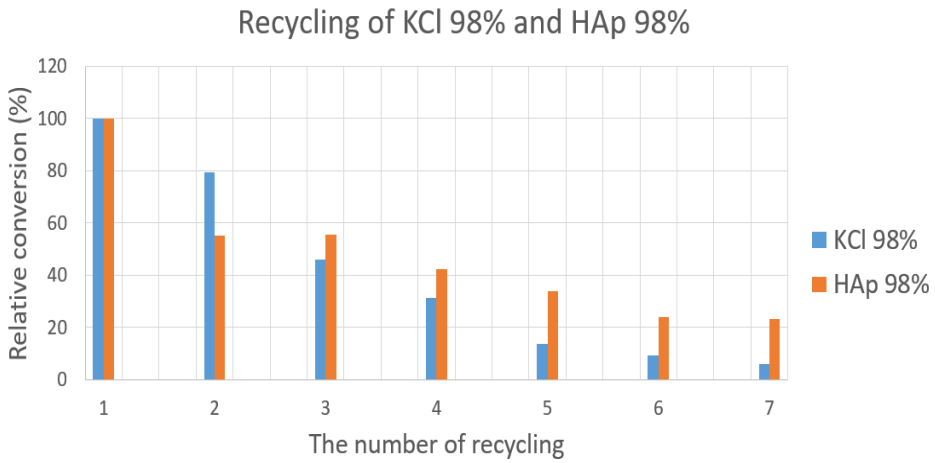


Figure 4. Recycling of KCl 98%, and HAp 98%.

3.3 Experimental Section

General methods

Protease from *Bacillus licheniformis* (subtilisin, ≥2.4 U/g), and hydroxyapatite were purchased from Sigma-Aldrich. All other chemicals were purchased from Sigma-Aldrich, Acros, and TCI. The analyses of gas chromatography was carried out by Agilent 6890N with a chiral capillary column (Cyclosil-B 30 m × 0.25 mm). The FE-SEM images were obtained by JSM-7500F (Jeol, Japan). XRD patterns were obtained by using a Bruker D8 Focus with a X-ray tube with a Cu target. IR was obtained by using Thermo Scientific Nicolet iS50 FT-IR spectrometer.

Preparation of a solution of protease from *Bacillus licheniformis*

A commercial subtilisin solution (1 mL) was desolved in a potassium-phosphate buffer (19 mL, pH 7.8, 10 mM), and the solution was filtered through a 0.8 μm filter. Then, the buffer of the solution was exchanged by a centrifugal device (Viva spin, GE Healthcare). After concentrated, the concentration of the subtilisin solution was determined by the Bradford assay.

Immobilization of -subtilisin on hydroxyapatite (HAp) or KCl

Immobilization was carried out by lyophilization. The subtilisin solution was mixed with a suspension of hydroxyapatite (98%, 95%, 90%, and 80% in water). For comparison, lyophilization with a KCl solution (98%) was carried out.

Transesterification of (±)-1-phenylethanol with vinyl butyrate

Immobilized subtilisin (125 mg) and molecular sives 4A (25 mg) were added in a mixture of 1 mmol of (±)-1-phenylethanol, 4 mmol of vinyl butyrate, and a solvent (2 mL, THF, *n*-hexane, diisopropyl ether, cyclohexane, and MTBE). The reaction mixture was stirred with magnetic bar and 25 °C.

A part (100 μL) of the reaction mixture was retrieved at predetermined time intervals of 10, 20, 30, 50, 60, 720, and 1440 min, and added in MTBE (1.2 mL). After removal of the immobilized enzymes by centrifugation, the solution was analyzed by gas chromatography (GC). The reaction conversion was calculated by the method presented by Sih and coworker s[22].

Gas Chromatography condition of transesterification of (±)-1-phenylethanol with vinyl butyrate

Gas Chromatography (GC) condition was 80 ℃ for 5 min, ramped with a rate of 2.5 ℃/min and holded at 120 ℃ for 15 min at the rate. And specific activity of synthesis of HA was 80 ℃ for 5 min, ramped with a rate of 1 ℃/min and holded at 95 ℃ for 60 min at the rate.

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22) Ching Shih Chen, Y. Fujimoto, Gary Girdaukas, & Charles J. Sih, *J. Am. Chem. Soc.* 1982, 104, 25, 7294-7299

Appendix

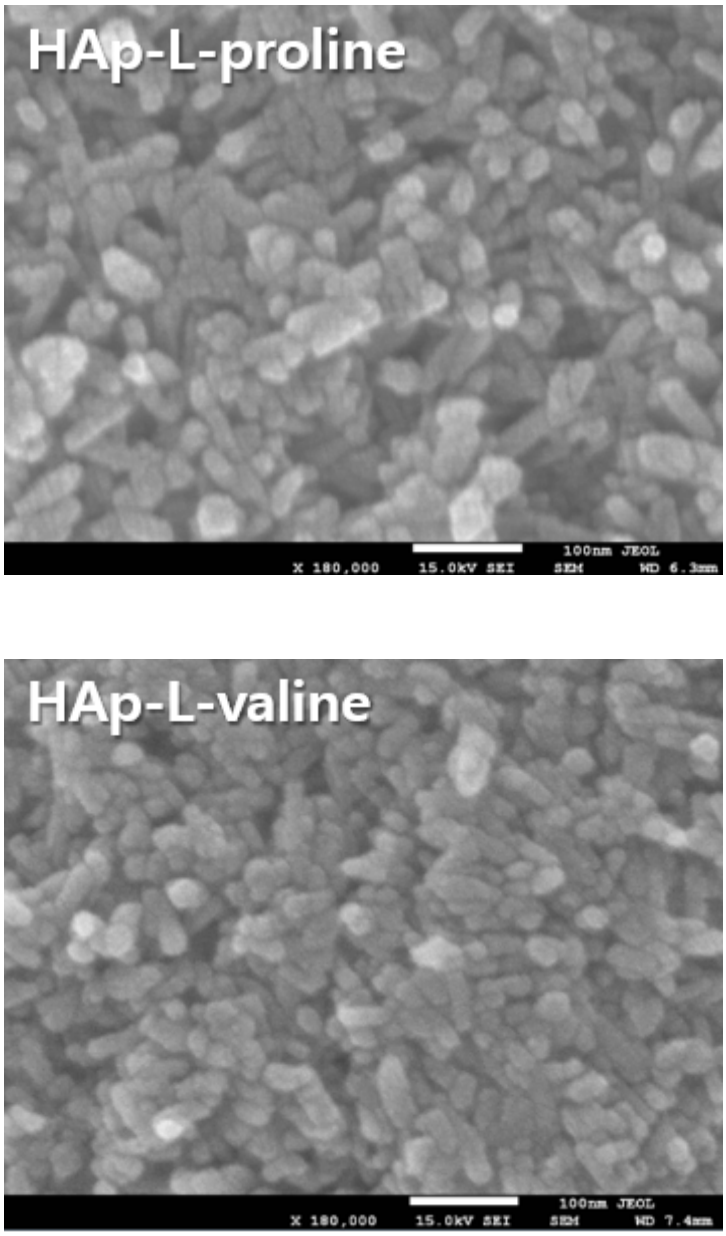


Figure 1. SEM of HAp-L-proline, and HAp-L-valine.

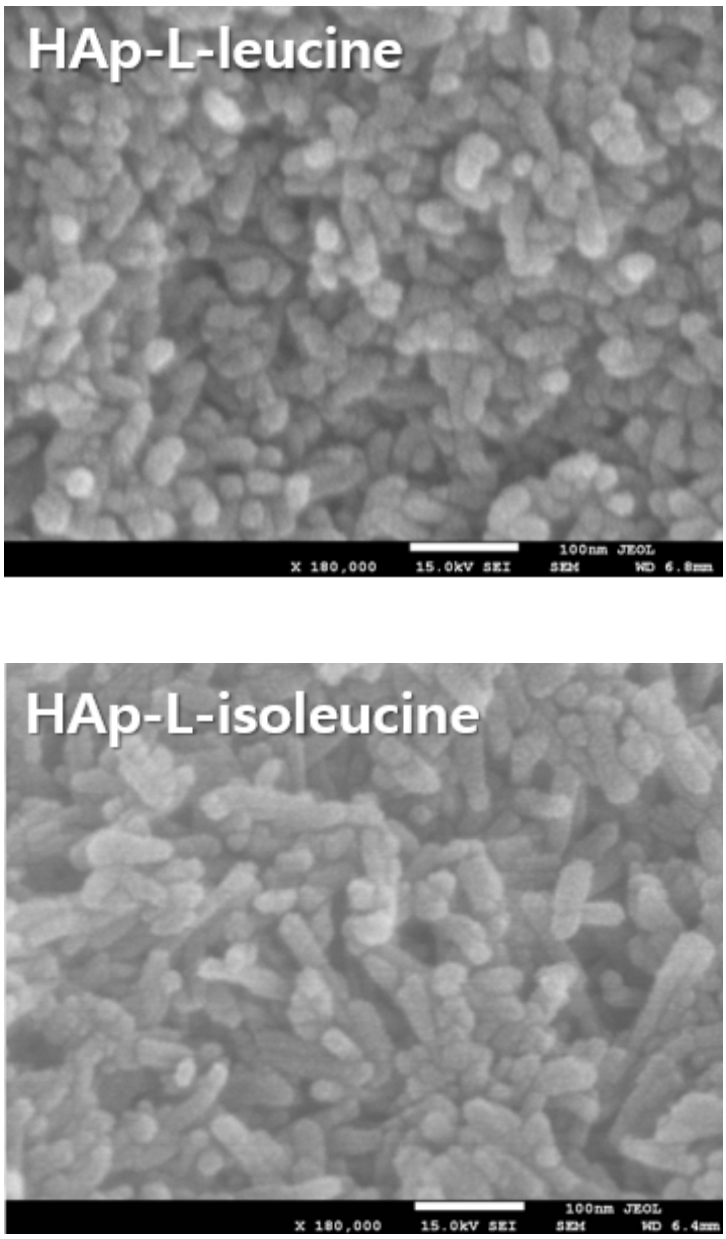


Figure 2. SEM of HAp-L-leucine, and HAp-L-isoleucine.

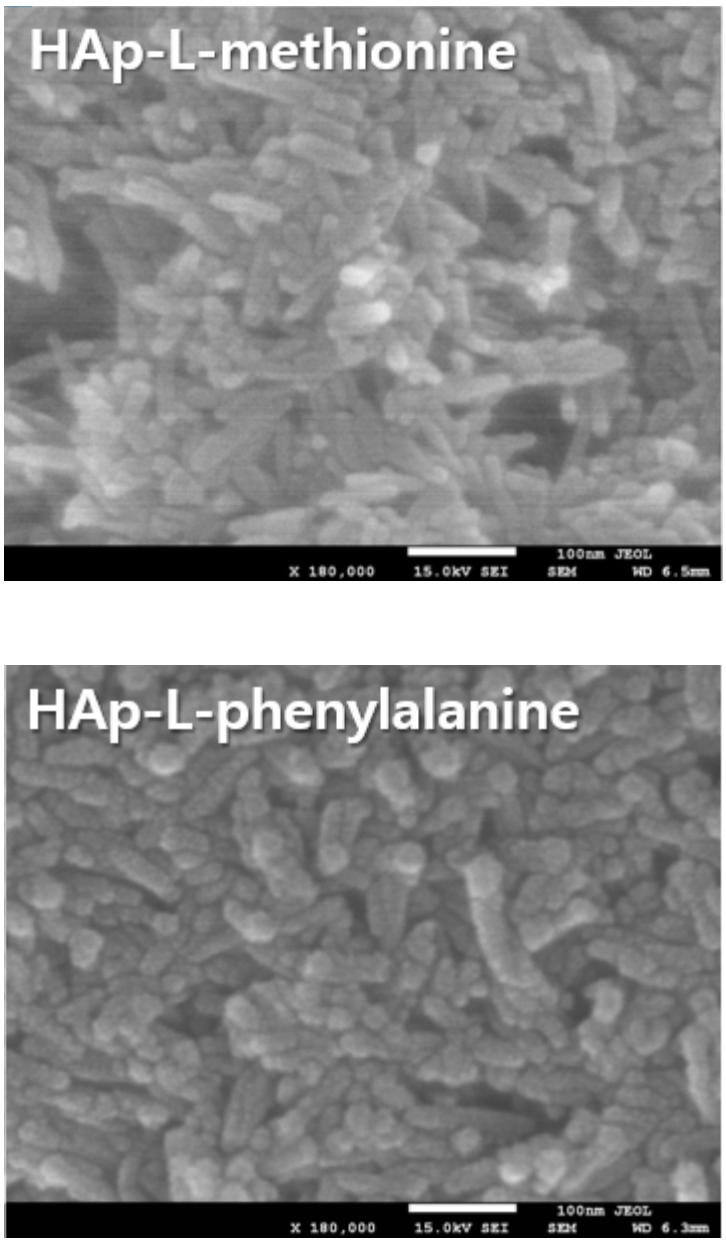


Figure 3. SEM of HAp-L-methionine, and HAp-L-phenylalanine.

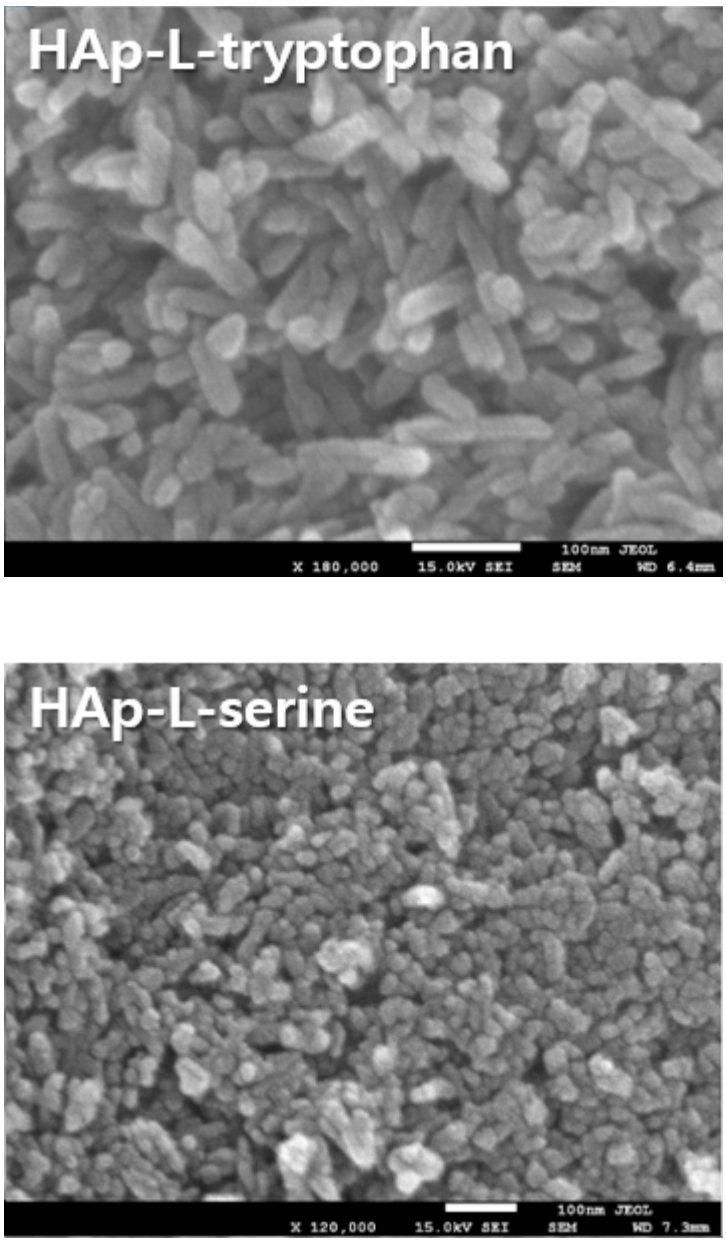


Figure 4. SEM of HAp-L-tryptophan, and HAp-L-serine.

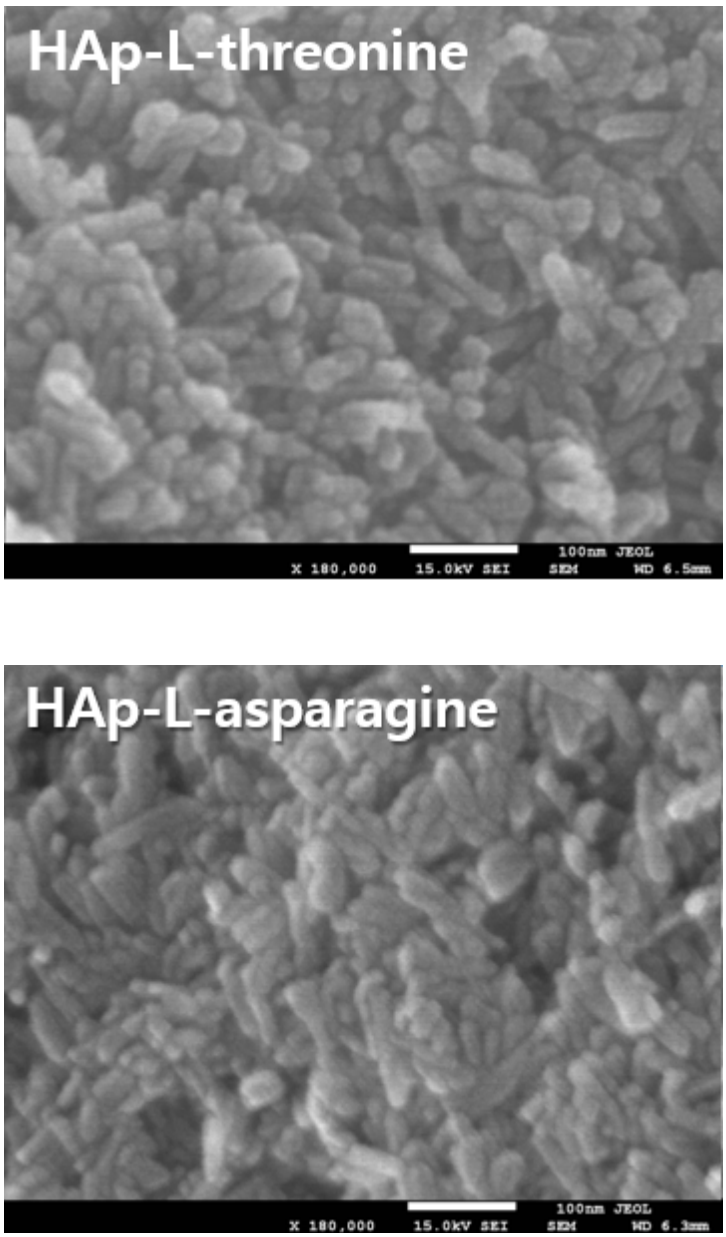


Figure 5. SEM of HAp-L-threonine, and HAp-L-asparagine.

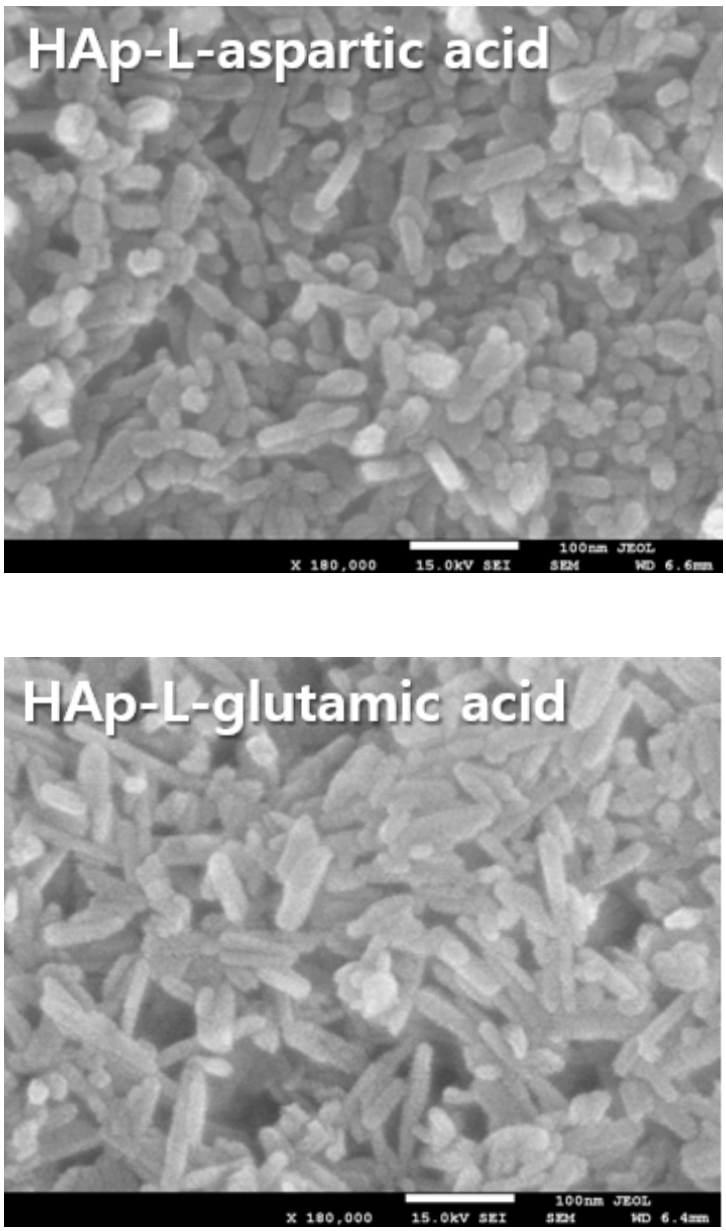


Figure 6. SEM of HAp-L-aspartic acid, and HAp-L-glutamic acid.

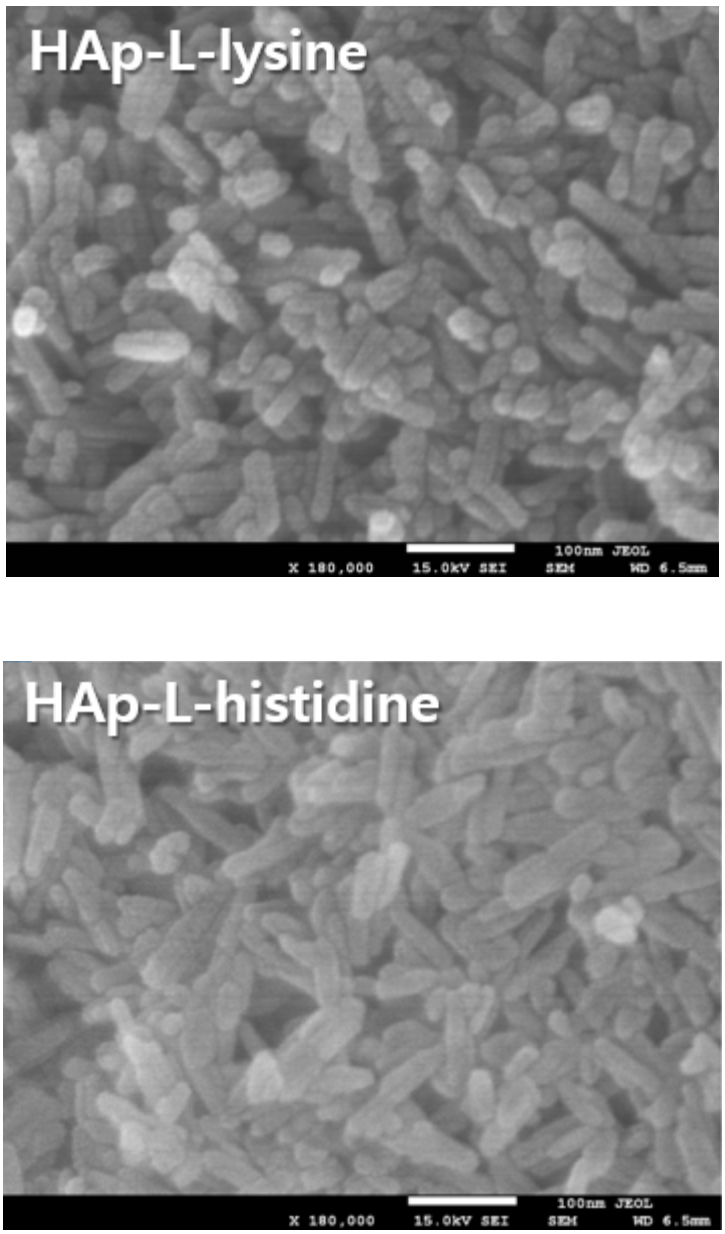


Figure 7. SEM of HAp-L-lysine, and HAp-L-histidine.

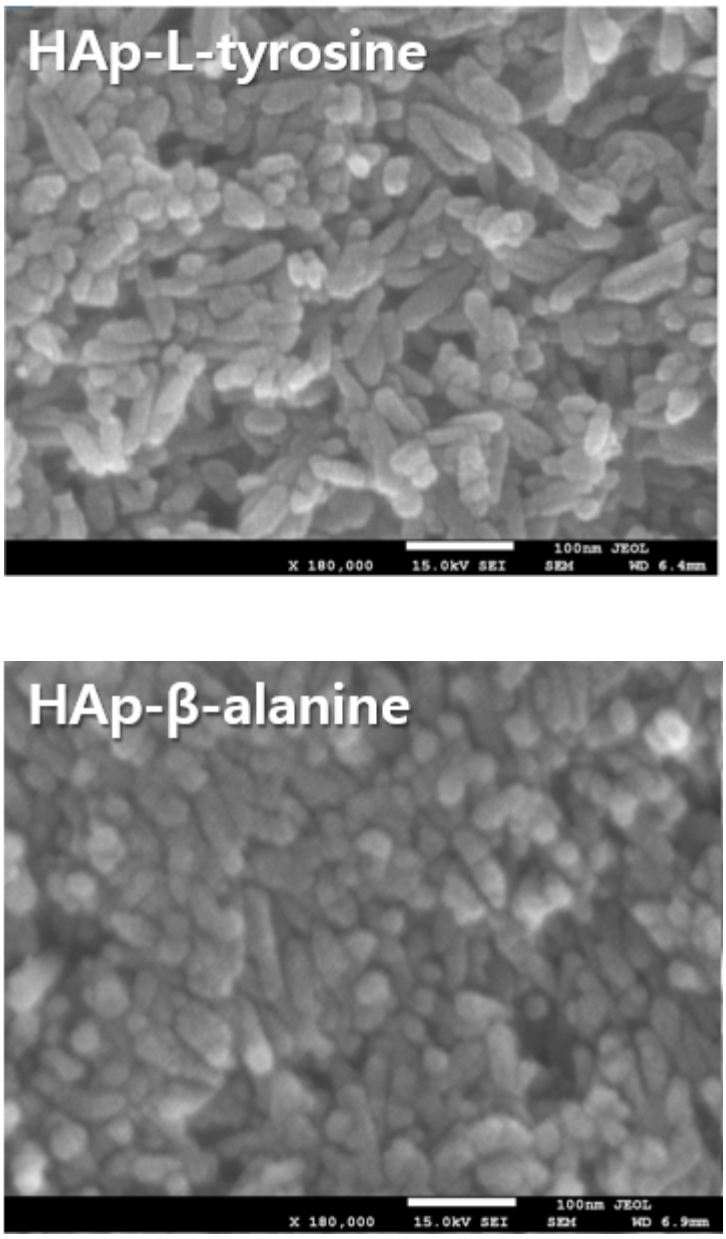


Figure 8. SEM of HAp-L-tyrosine and HAp-β-alanine.

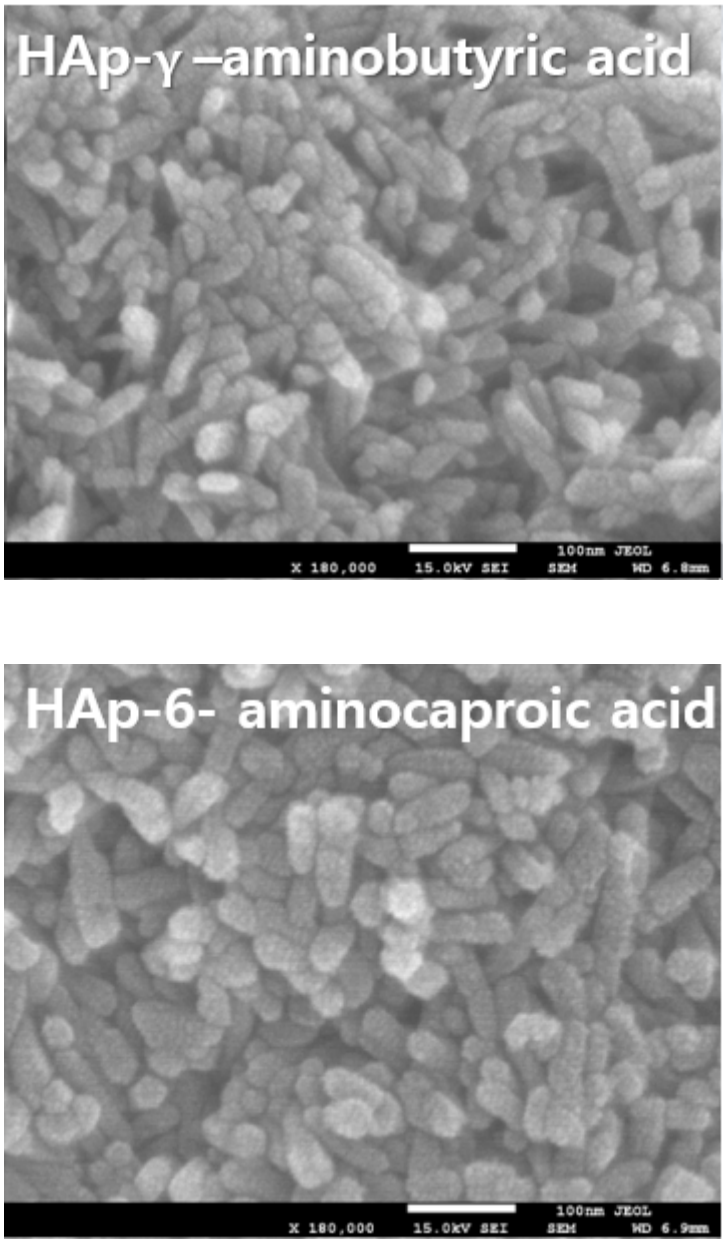


Figure 9. SEM of γ-aminiobutyric acid and HAp-L-6-aminocaproic acid.

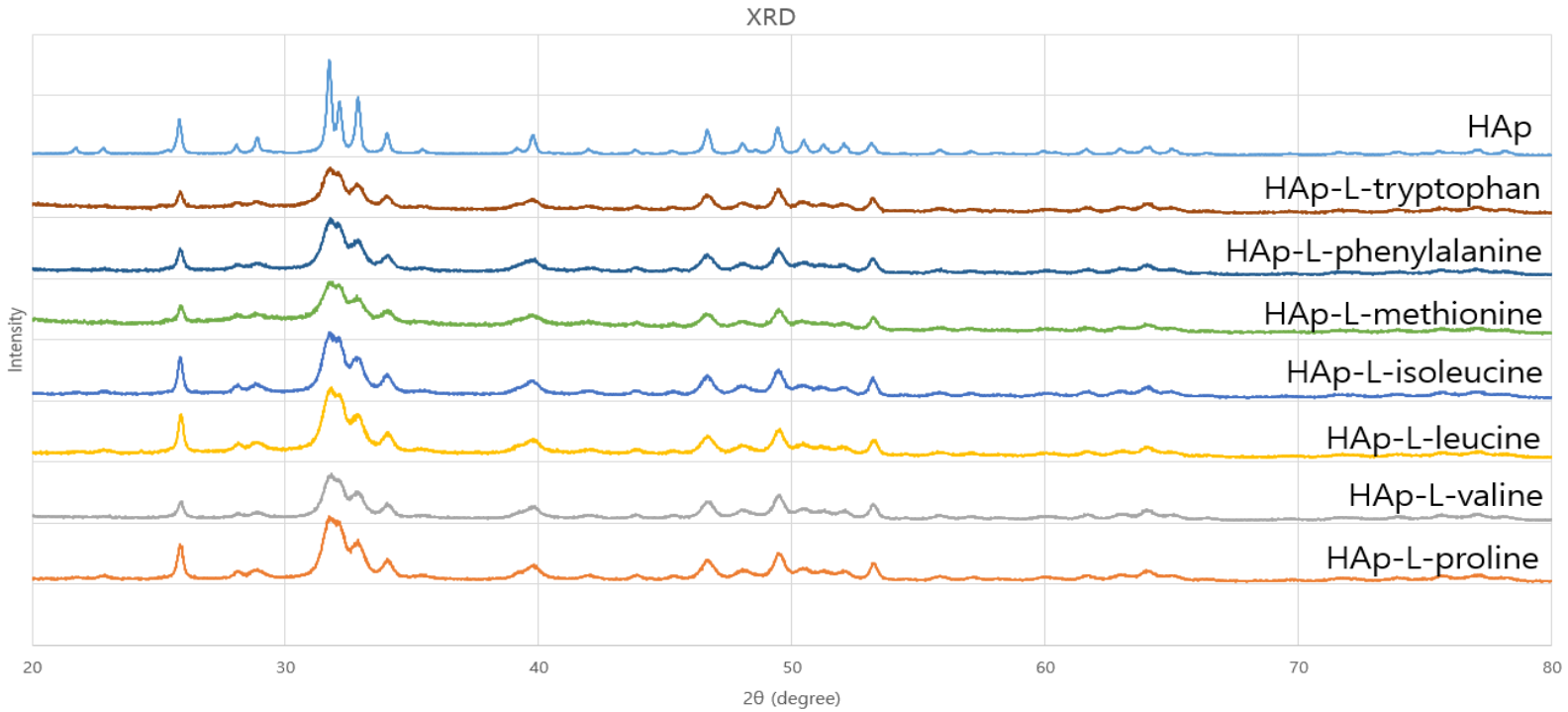


Figure 10. XRD of HAp-amino acid.

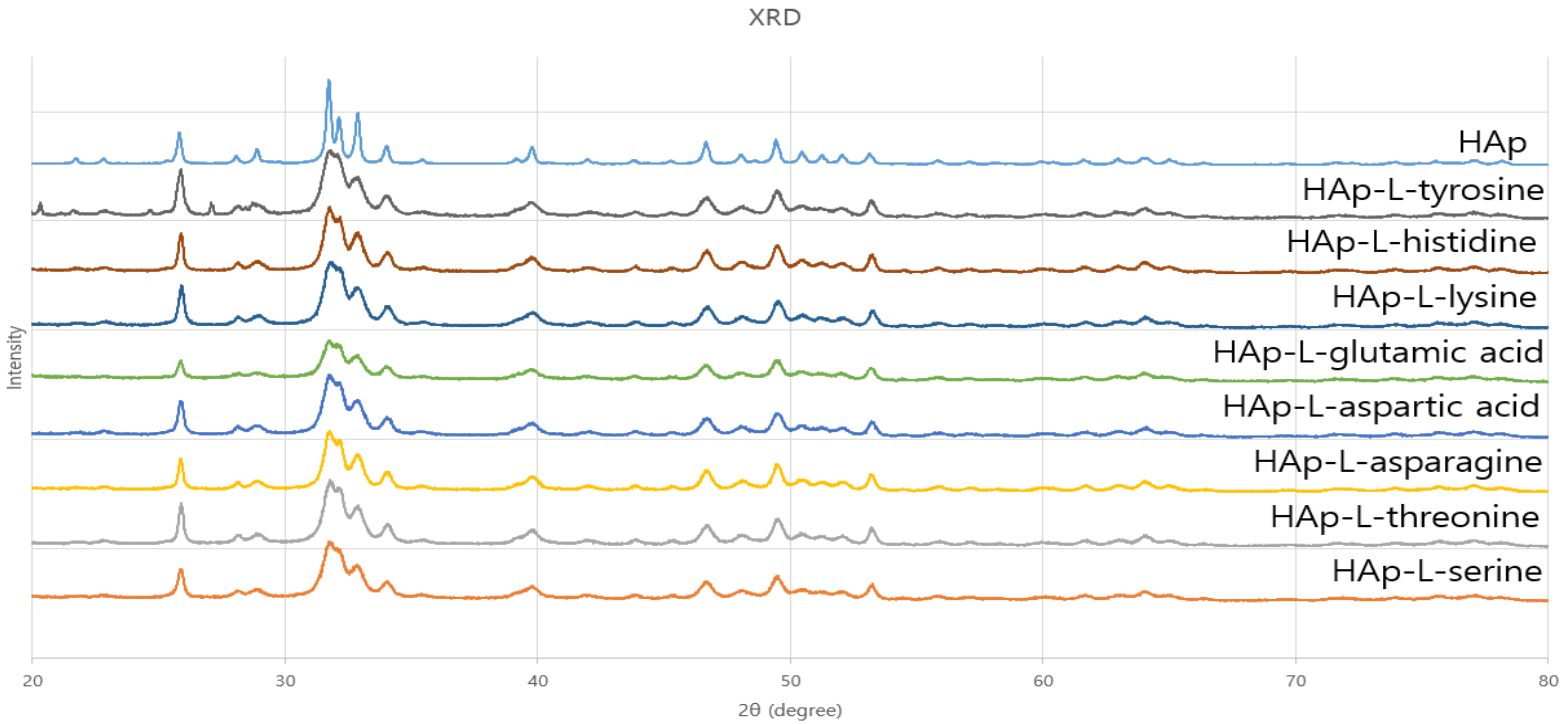


Figure 10 continued. XRD of HAp-amino acid.

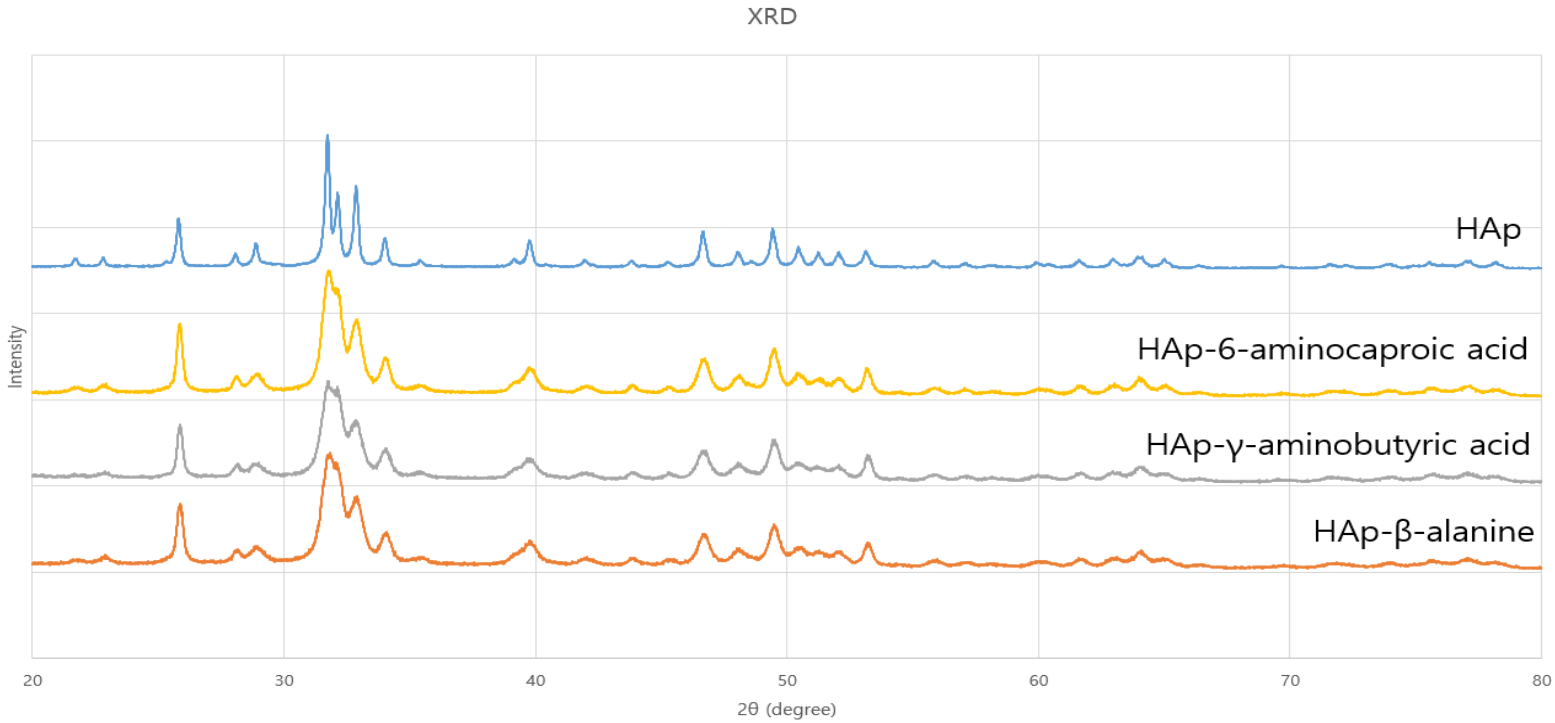


Figure 10 continued. XRD of HAp-amino acid.

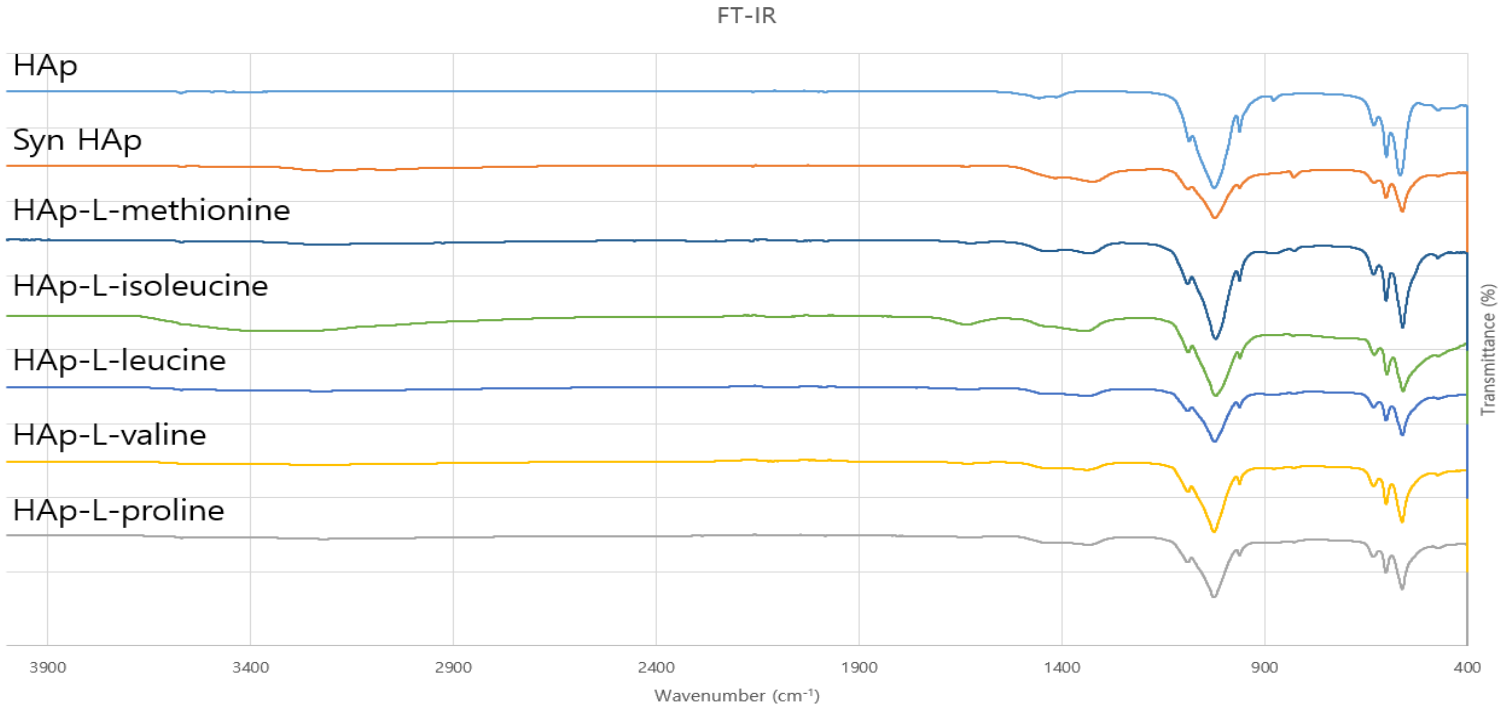


Figure 11. IR of HAp-amino acid.

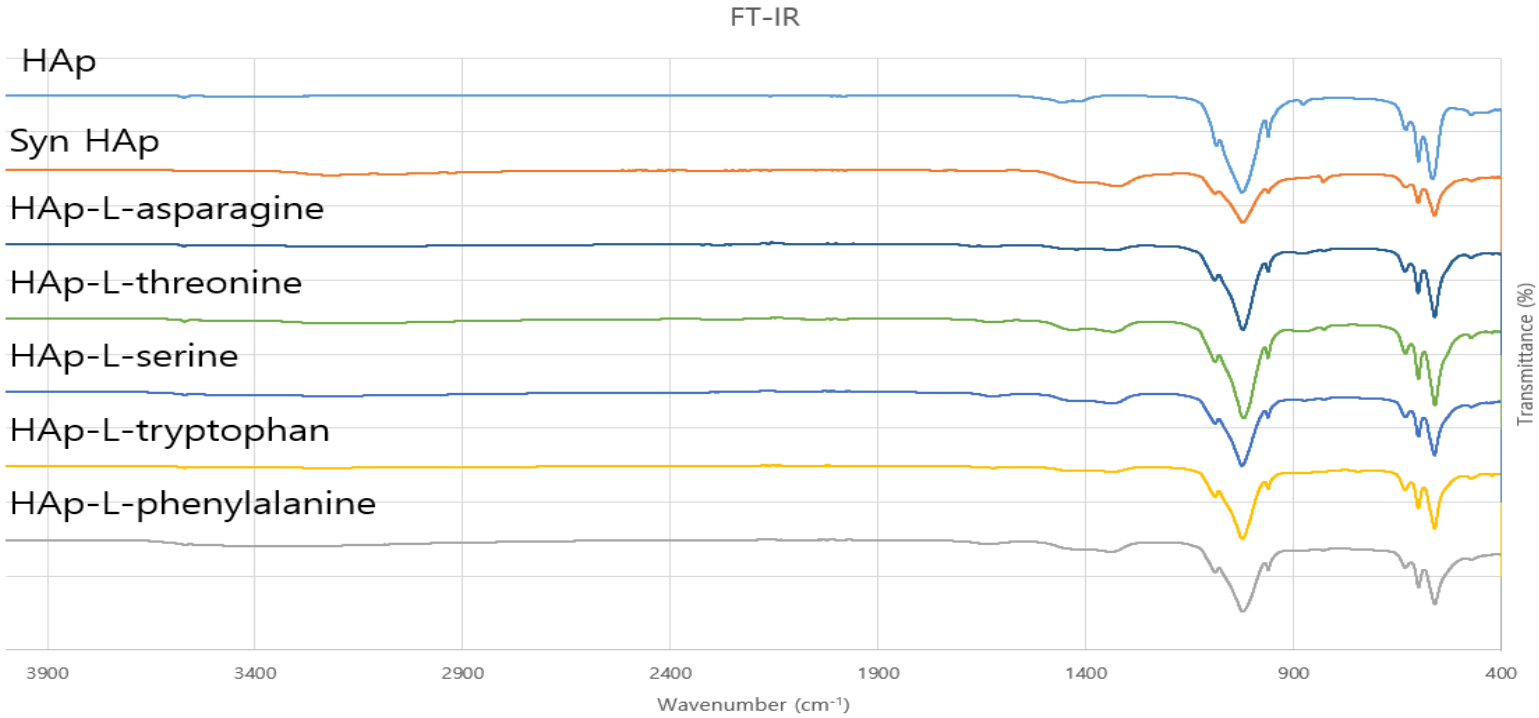


Figure 11 continued. IR of HAp-amino acid.

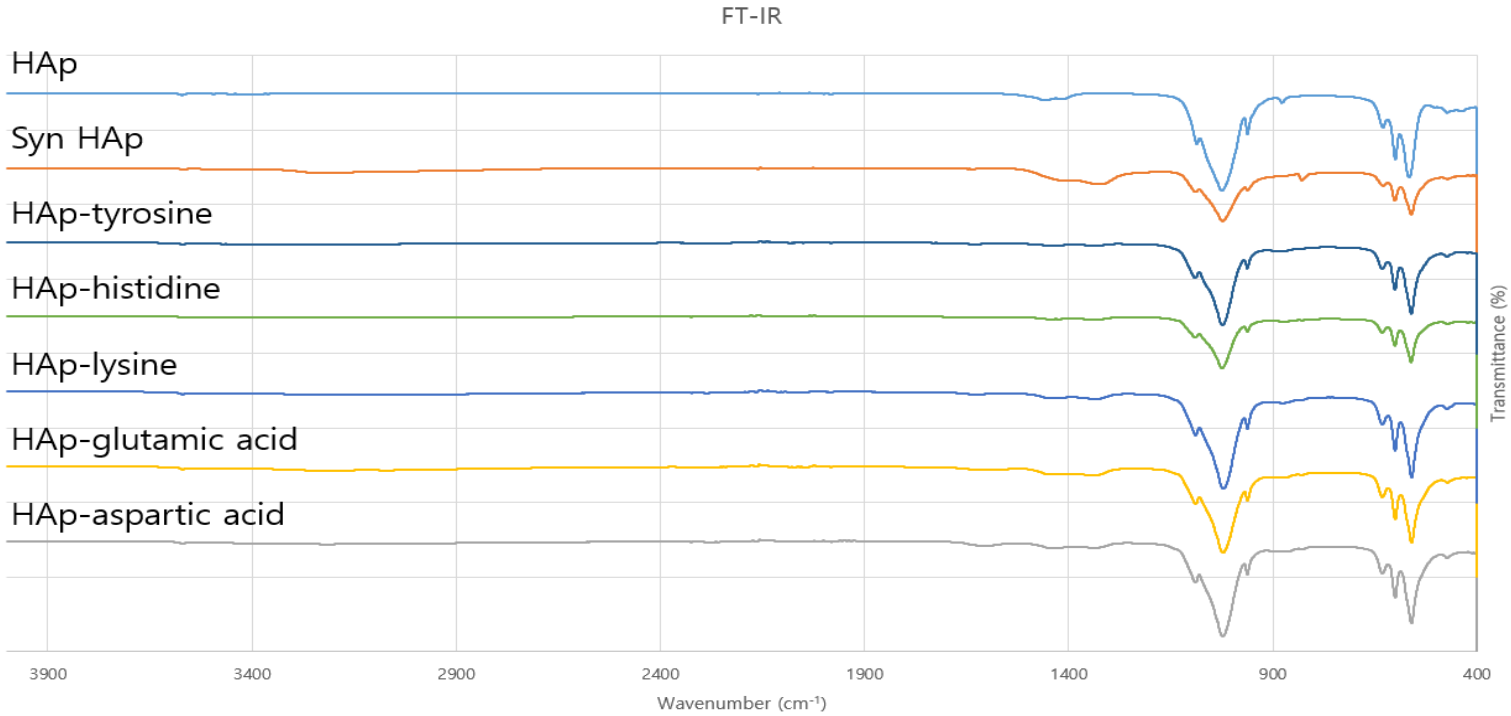


Figure 11 continued. IR of HAp-amino acid.

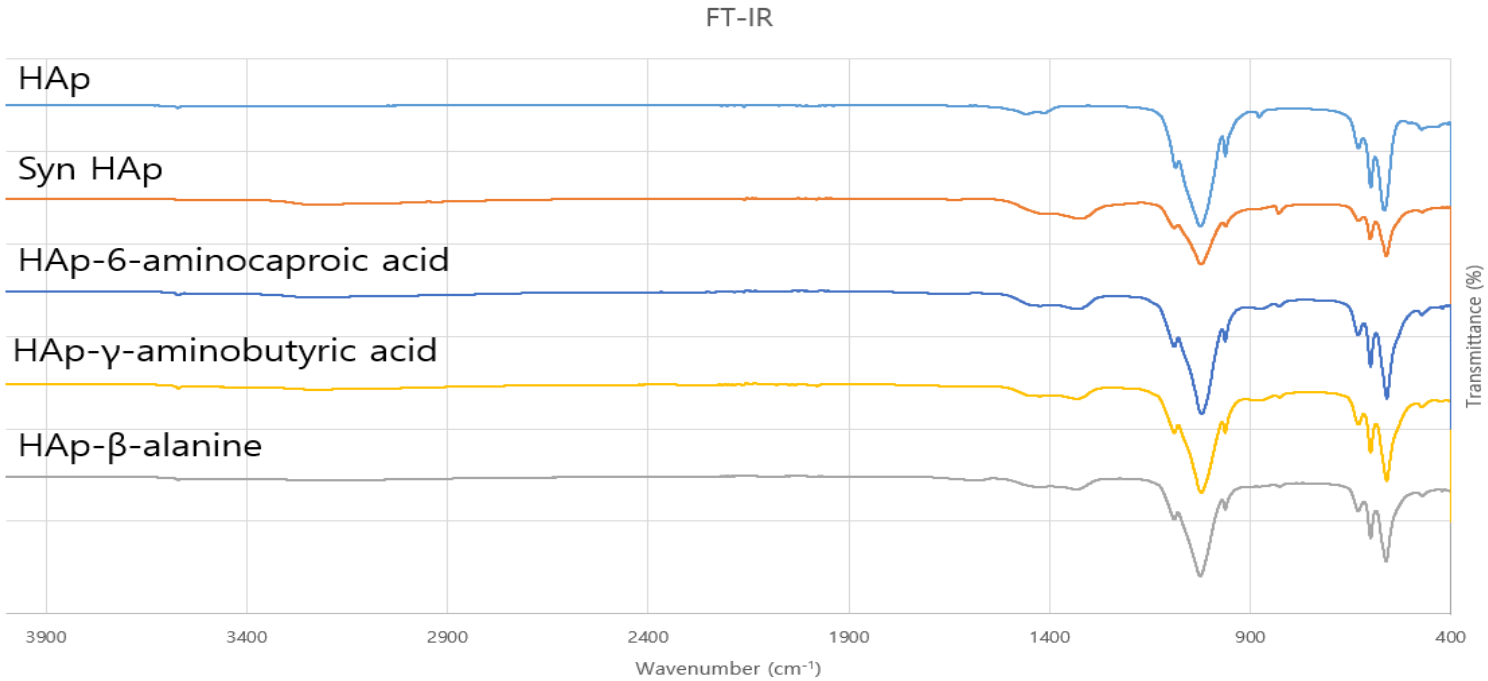


Figure 11 continued. IR of HAp-amino acid.

Only glycine

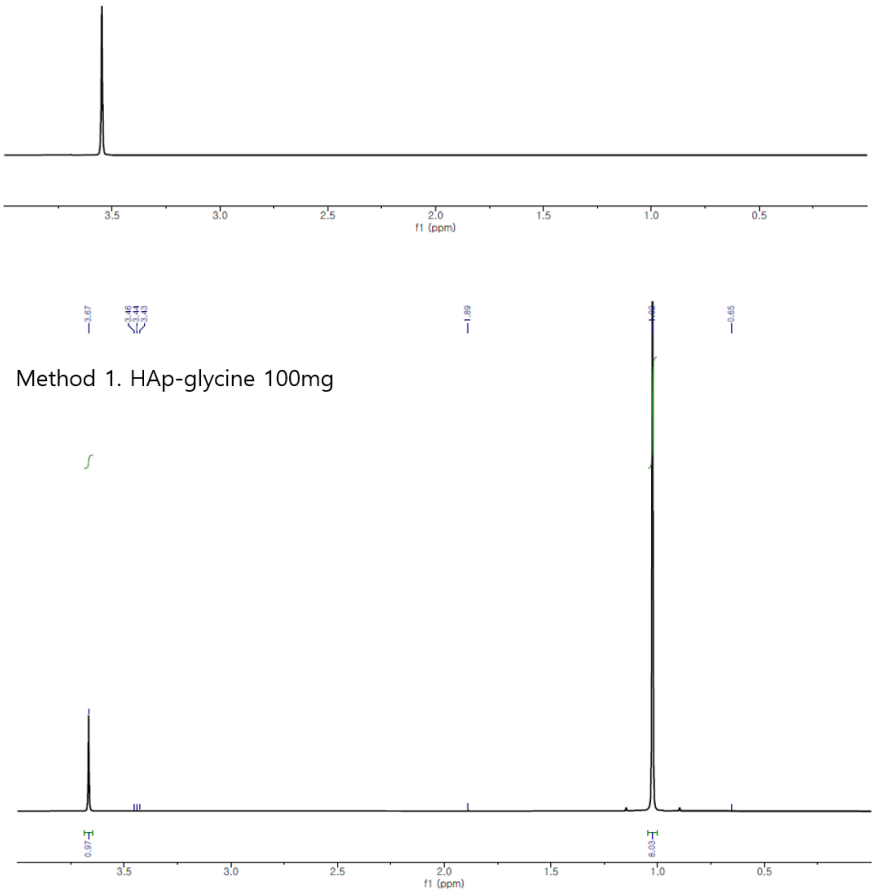
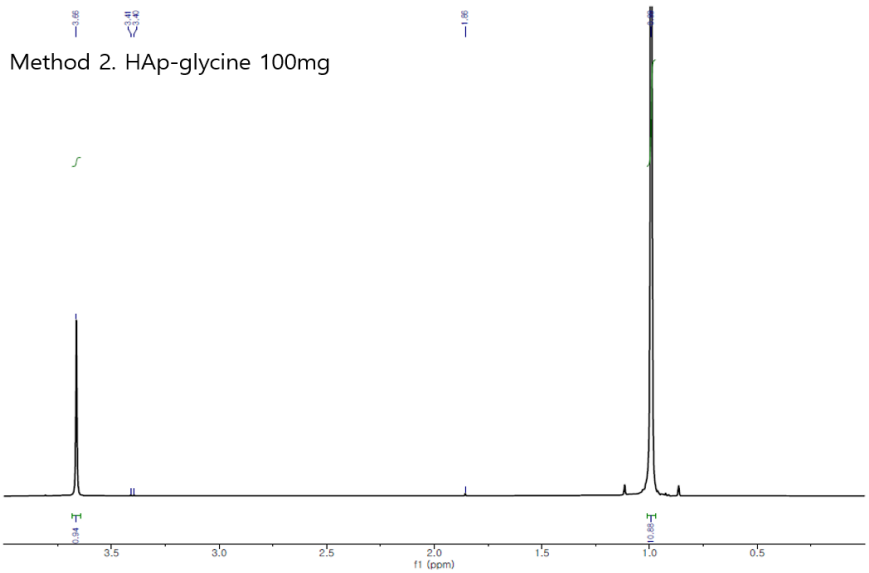


Figure 12. NMR of only glycine and HAp-glycine (method 1).



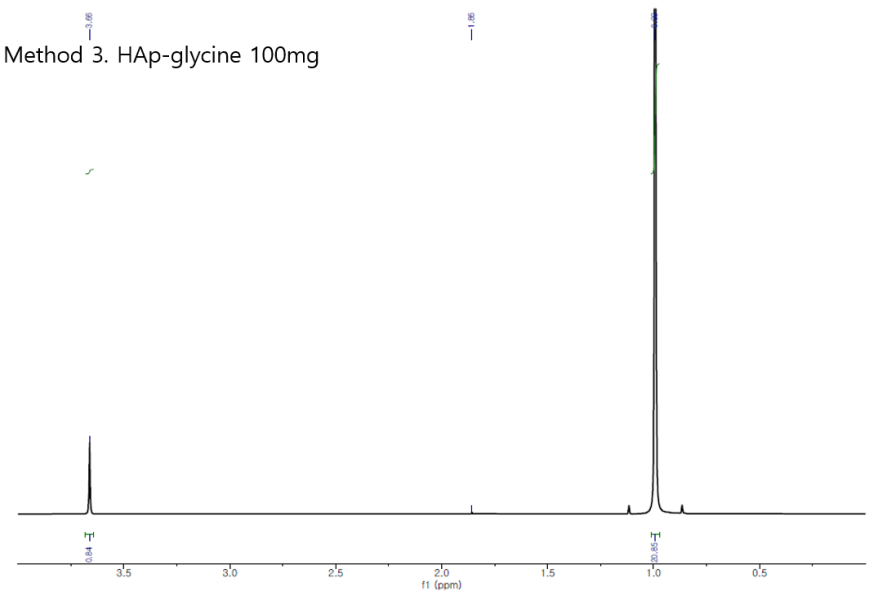


Figure 13. NMR of HAp-glycine (method 2, 3).

Only proline

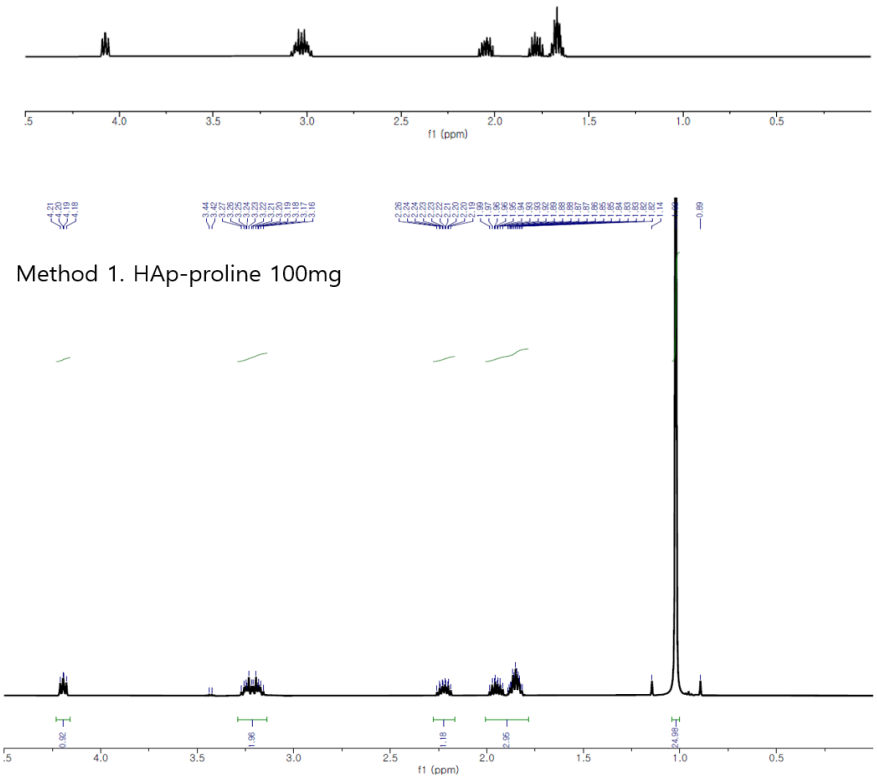


Figure 14. NMR of only proline and HAp-proline (method 1).

Only valine

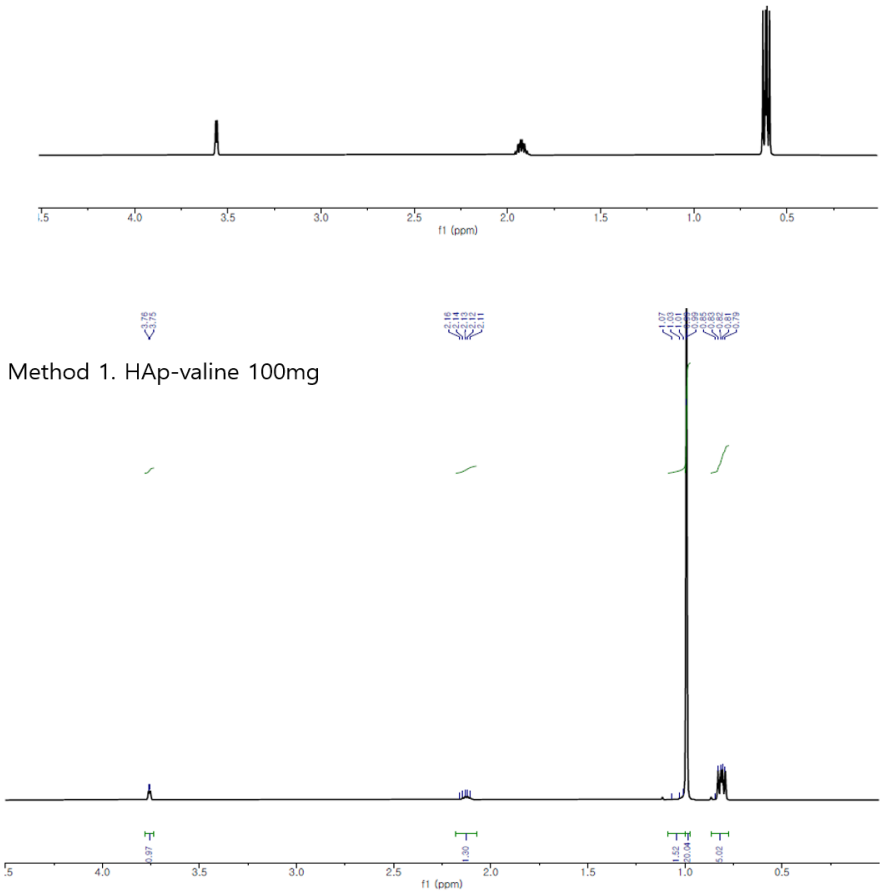


Figure 15. NMR of only valine and HAp-valine (method 1).

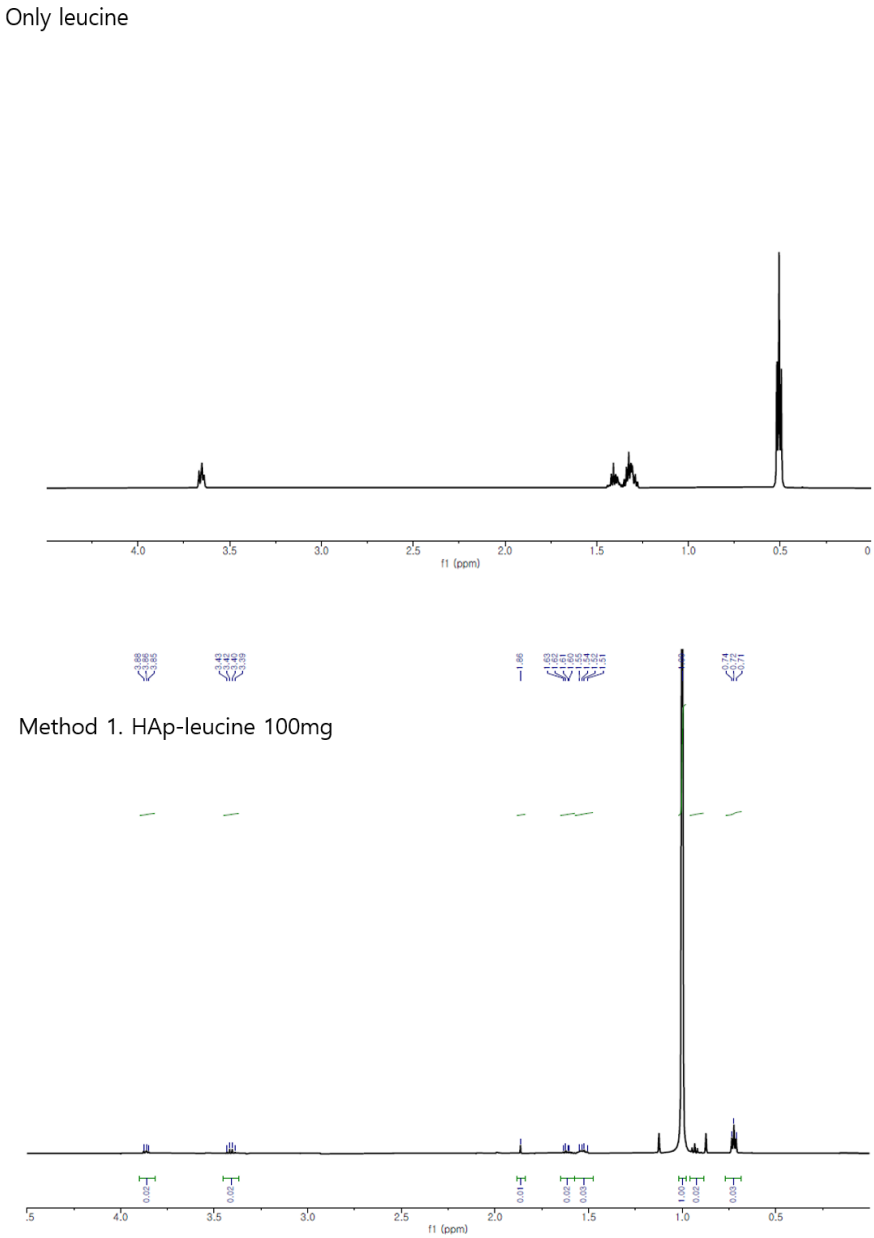


Figure 16. NMR of only leucine and HAp-leucine (method 1).

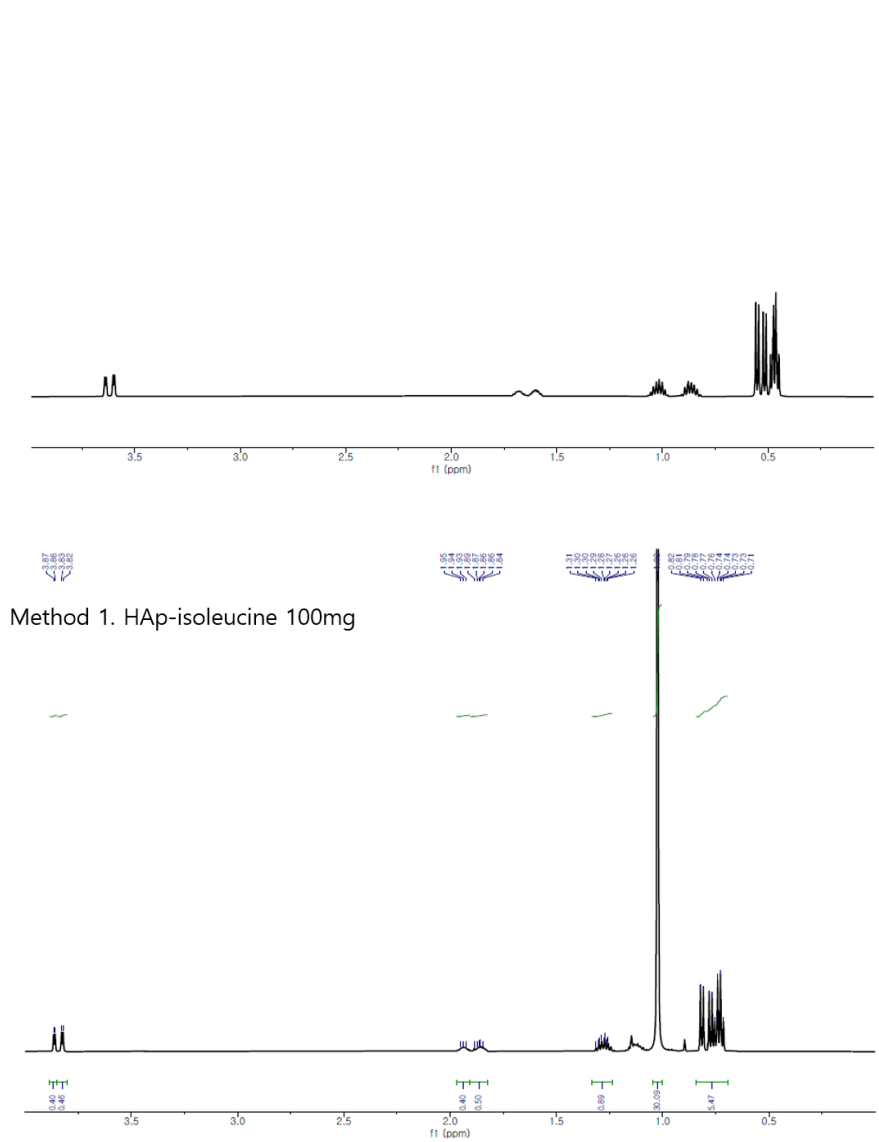


Figure 17. NMR of only isoleucine and HAp-isoleucine (method 1).

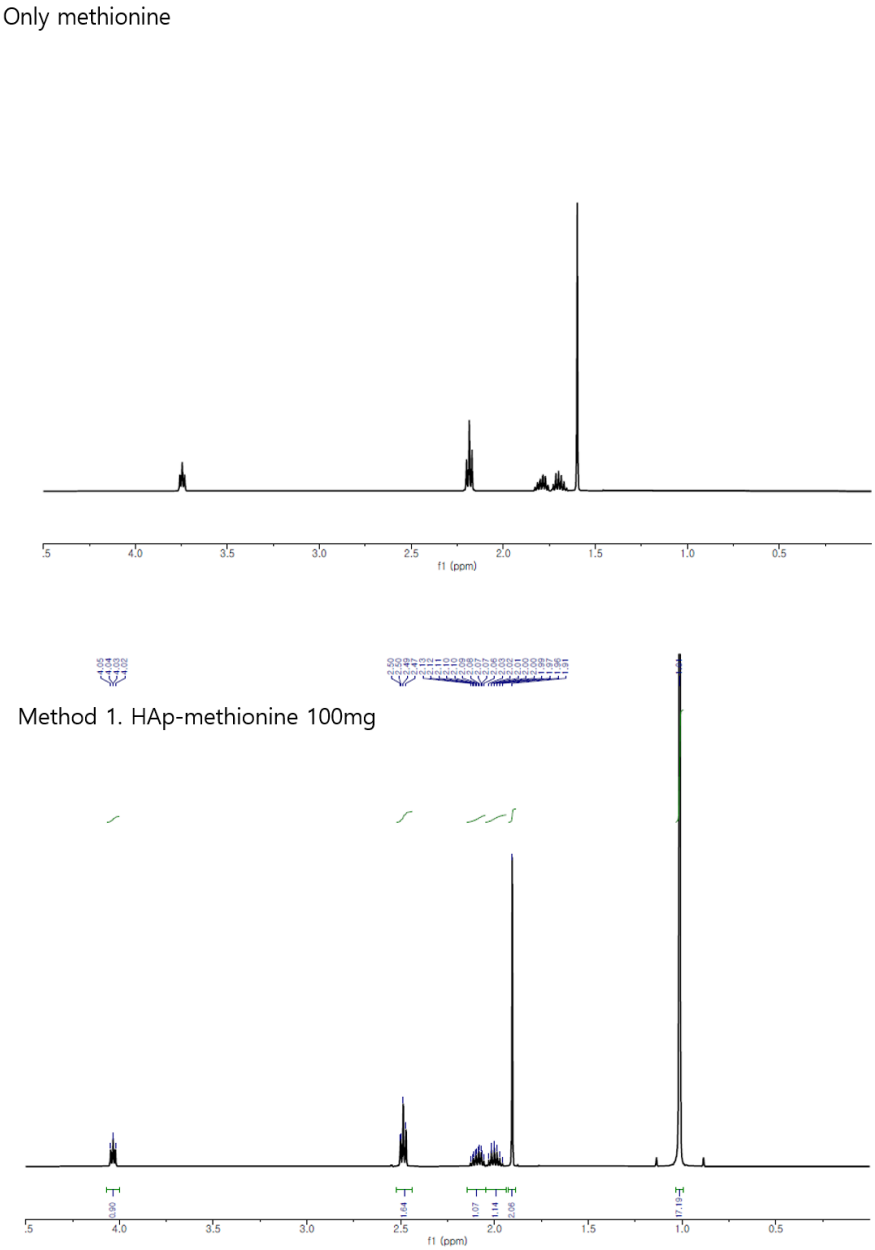


Figure 18. NMR of only methionine and HAp-methionine (method 1).

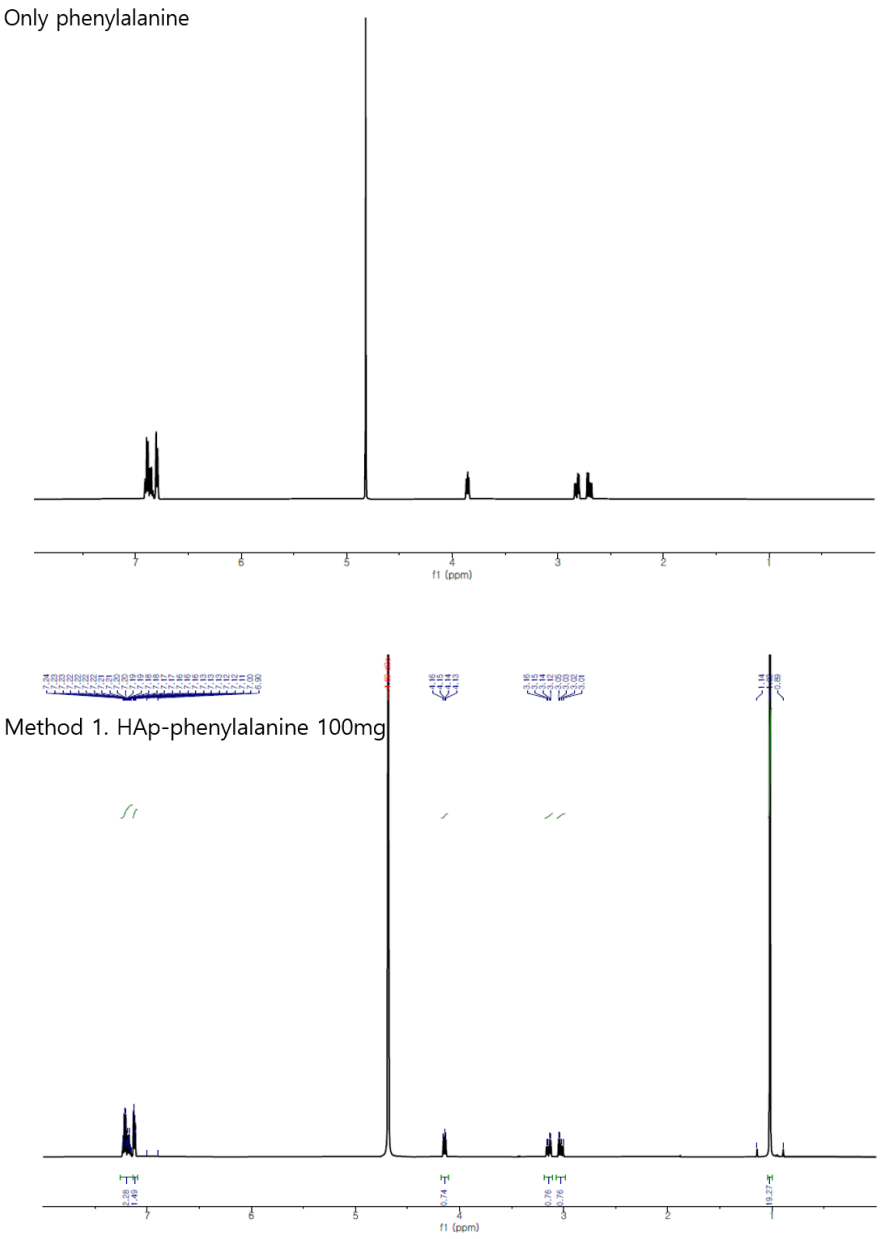


Figure 19. NMR of only phenylalanine and HAp-phenylalanine (method 1).

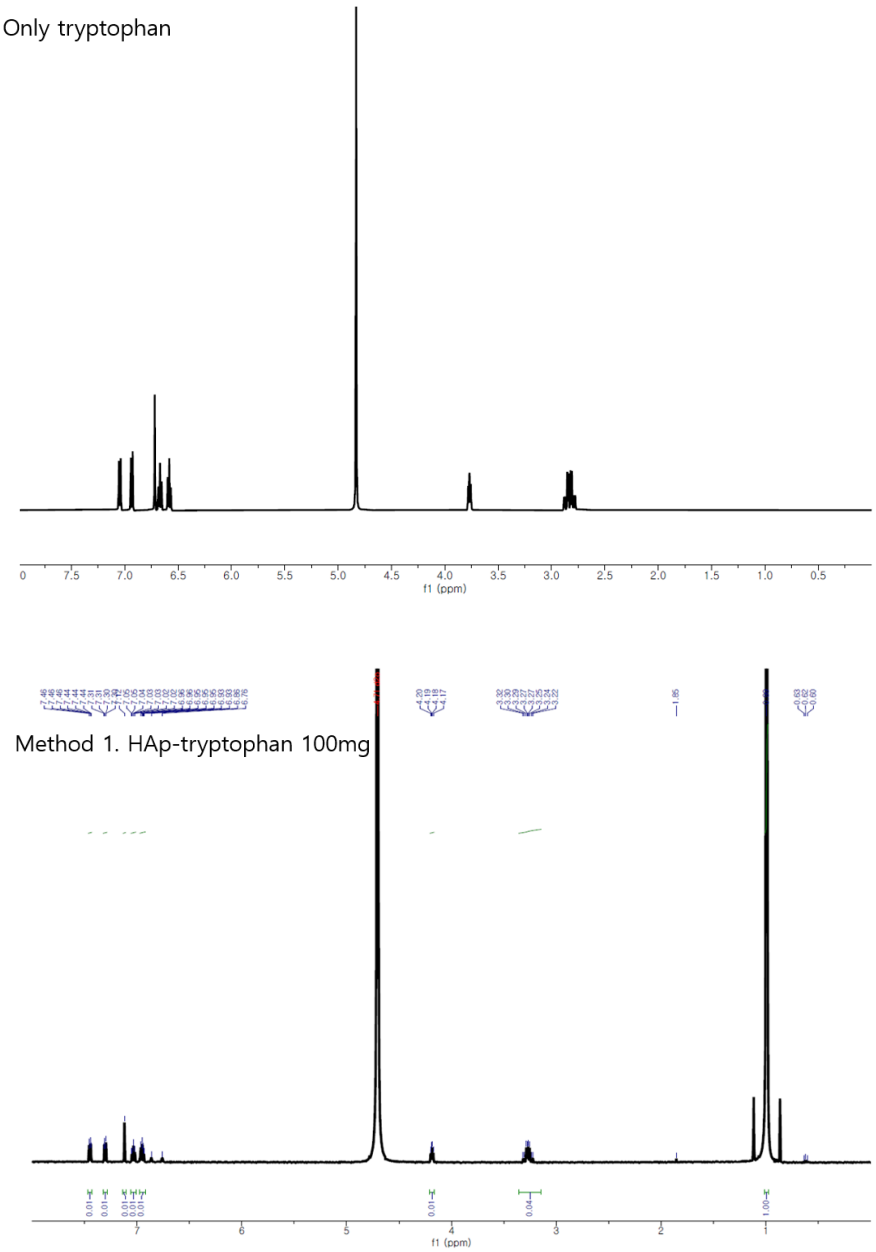


Figure 20. NMR of only tryptophan and HAp-tryptophan (method 1).

Only serine

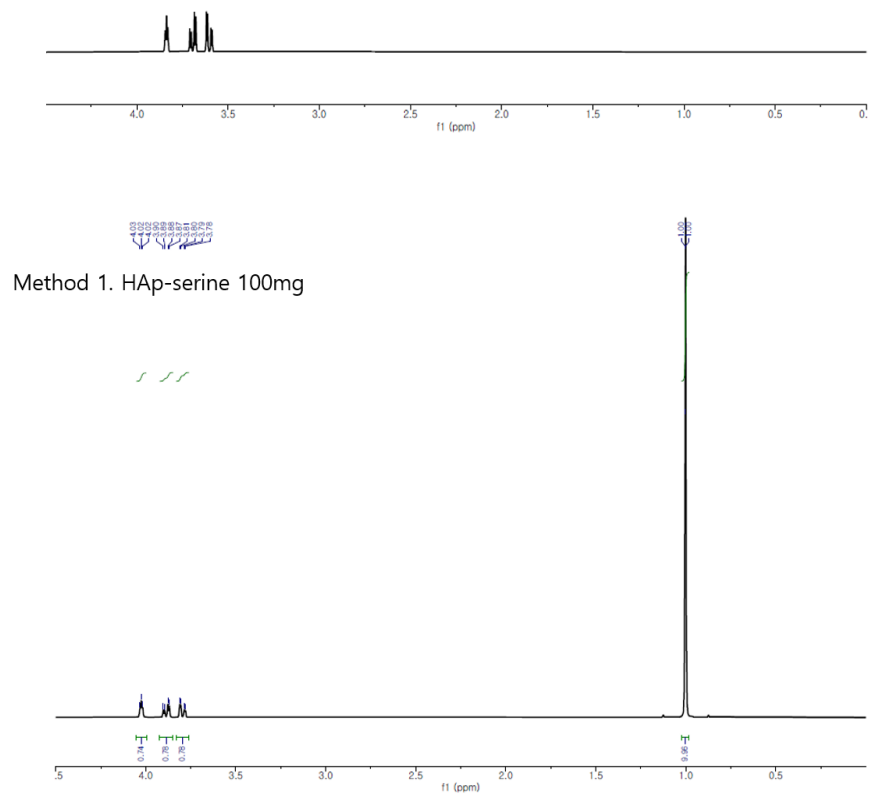
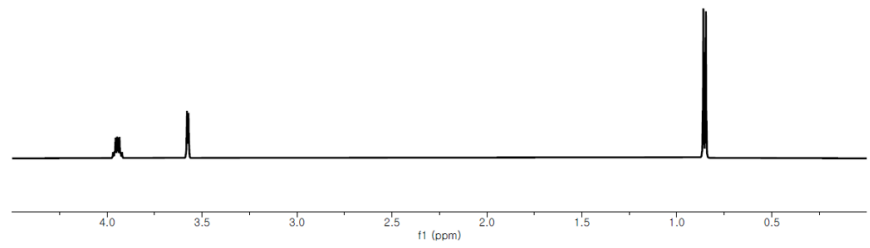


Figure 21. NMR of only serine and HAp-serine (method 1).

Only threonine



Method 1. HAp-threonine 100mg

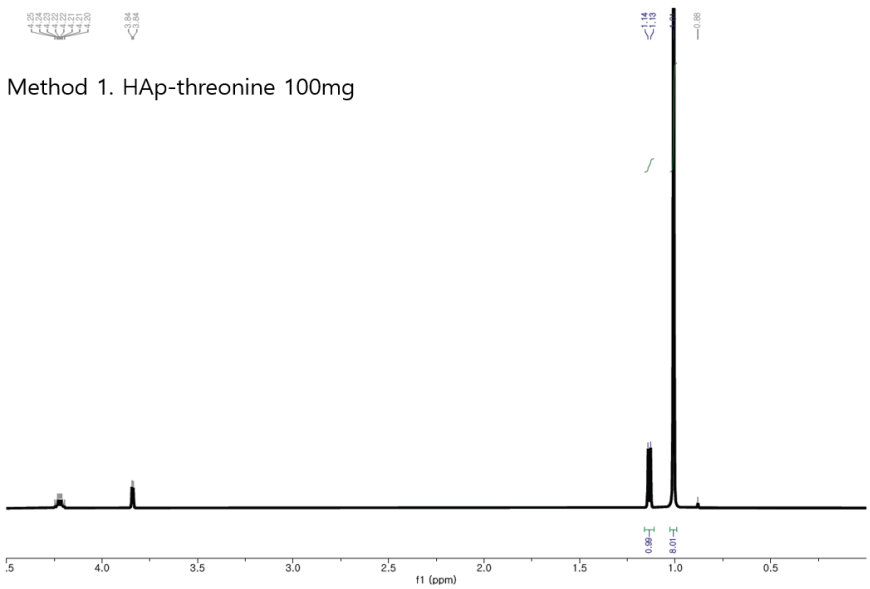


Figure 22. NMR of only threonine and HAp-threonine (method 1).

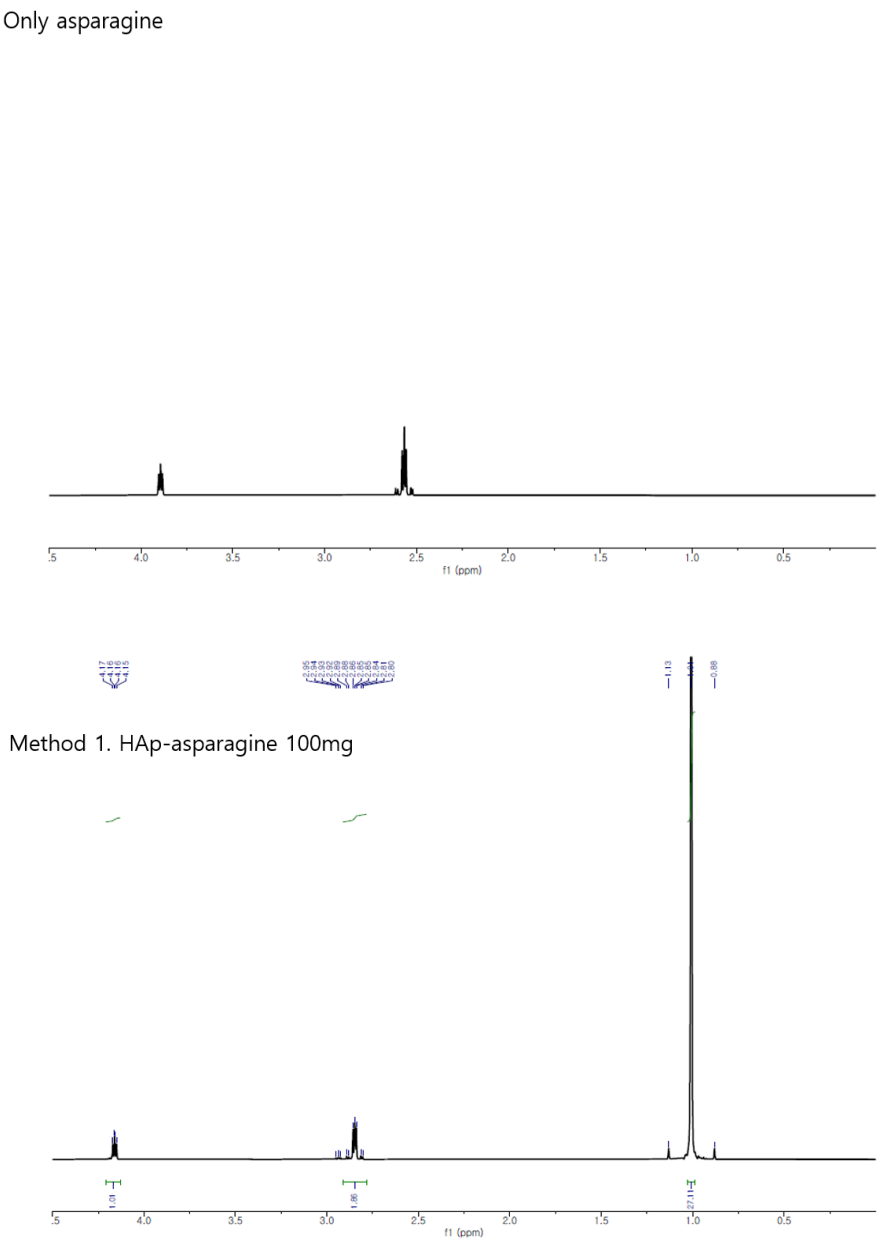


Figure 23. NMR of only asparagine and HAp-asparagine (method 1).

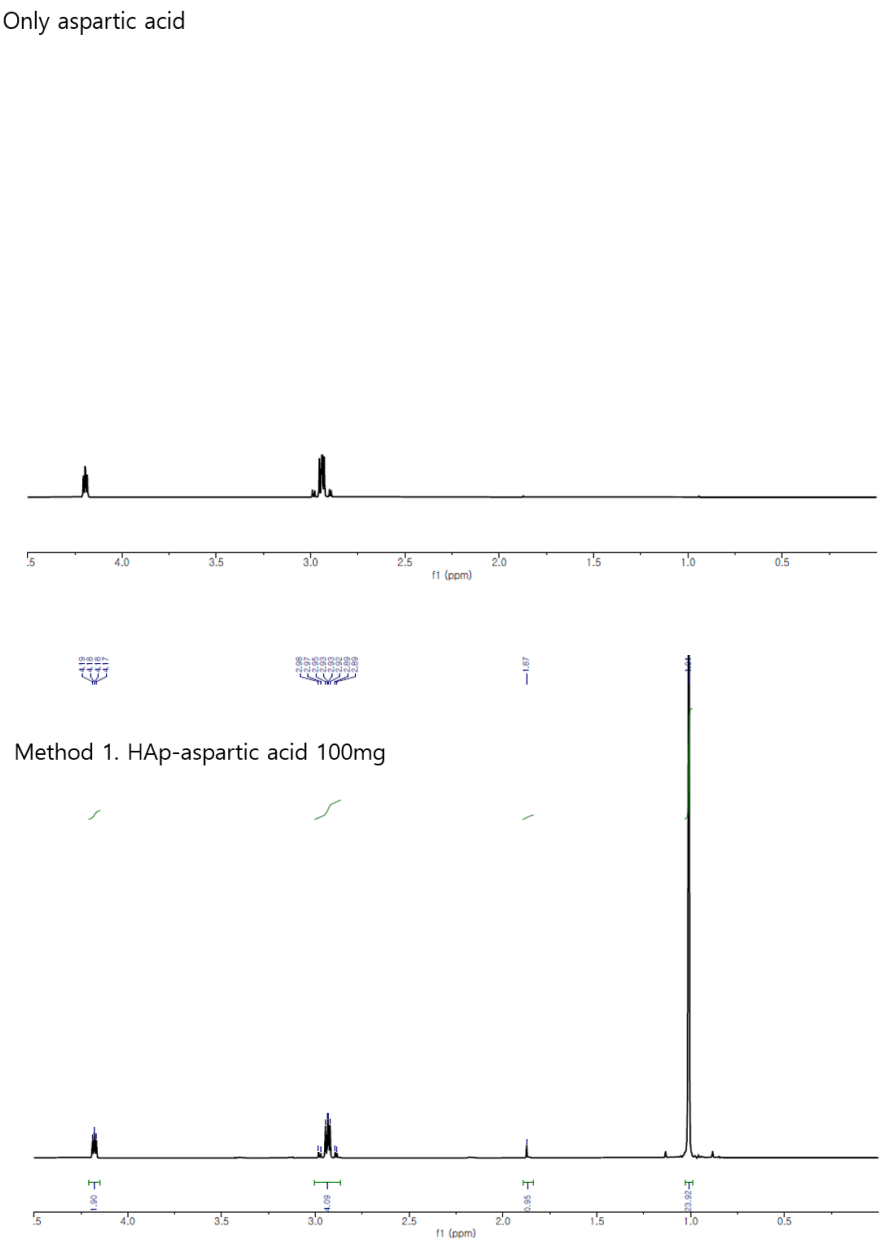


Figure 24. NMR of only aspartic acid and HAp-aspartic acid (method 1).

Only glutamic acid

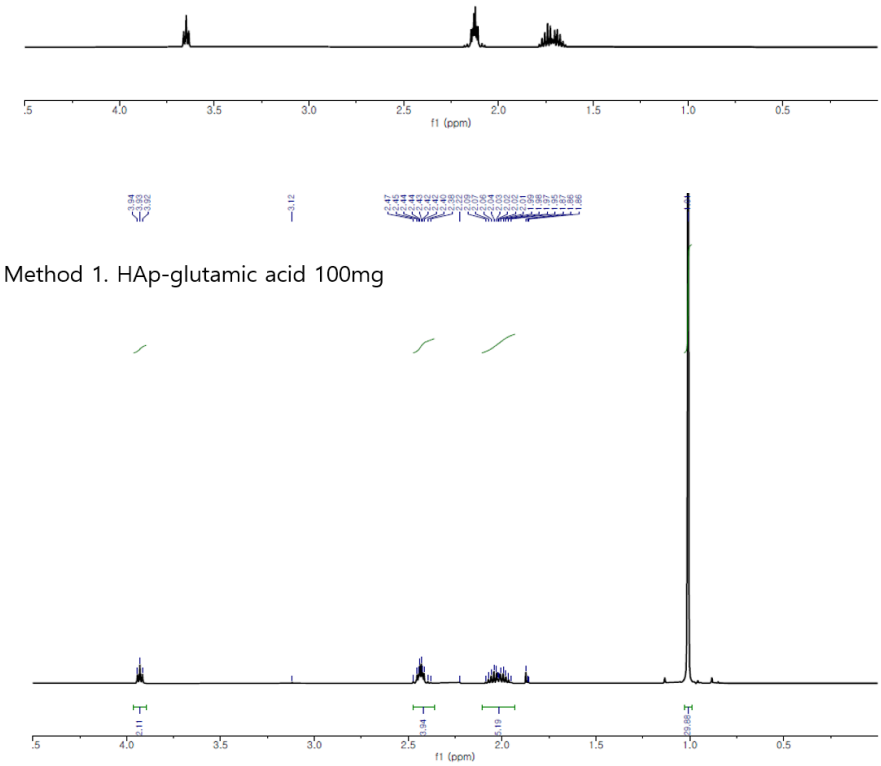


Figure 25. NMR of only glutamic acid and HAp-glutamic acid (method 1).

Only lysine

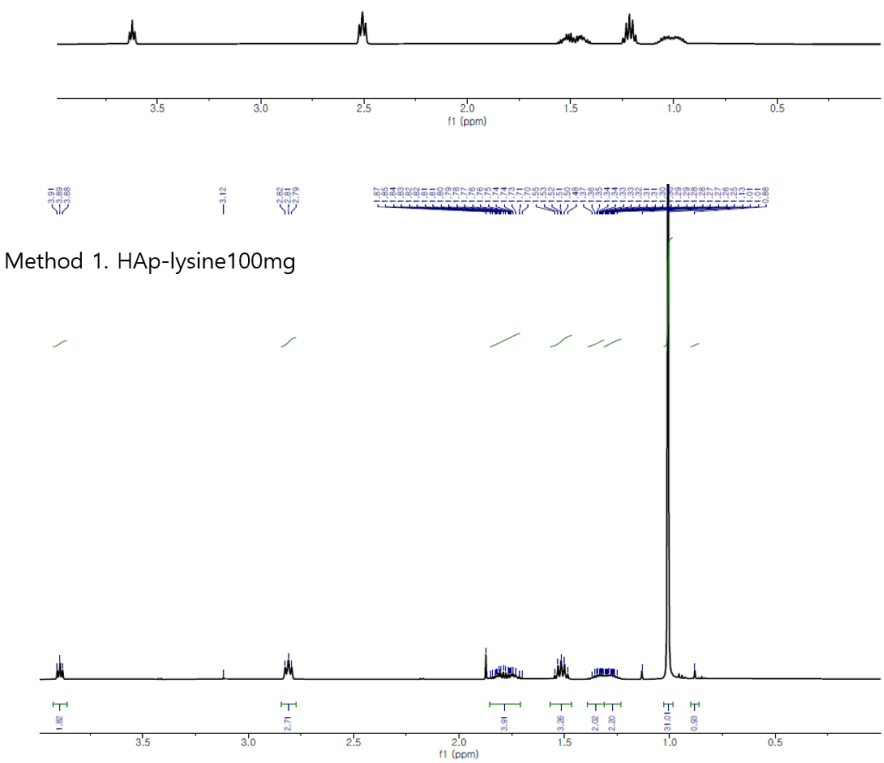


Figure 26. NMR of only lysine and HAp-lysine (method 1).

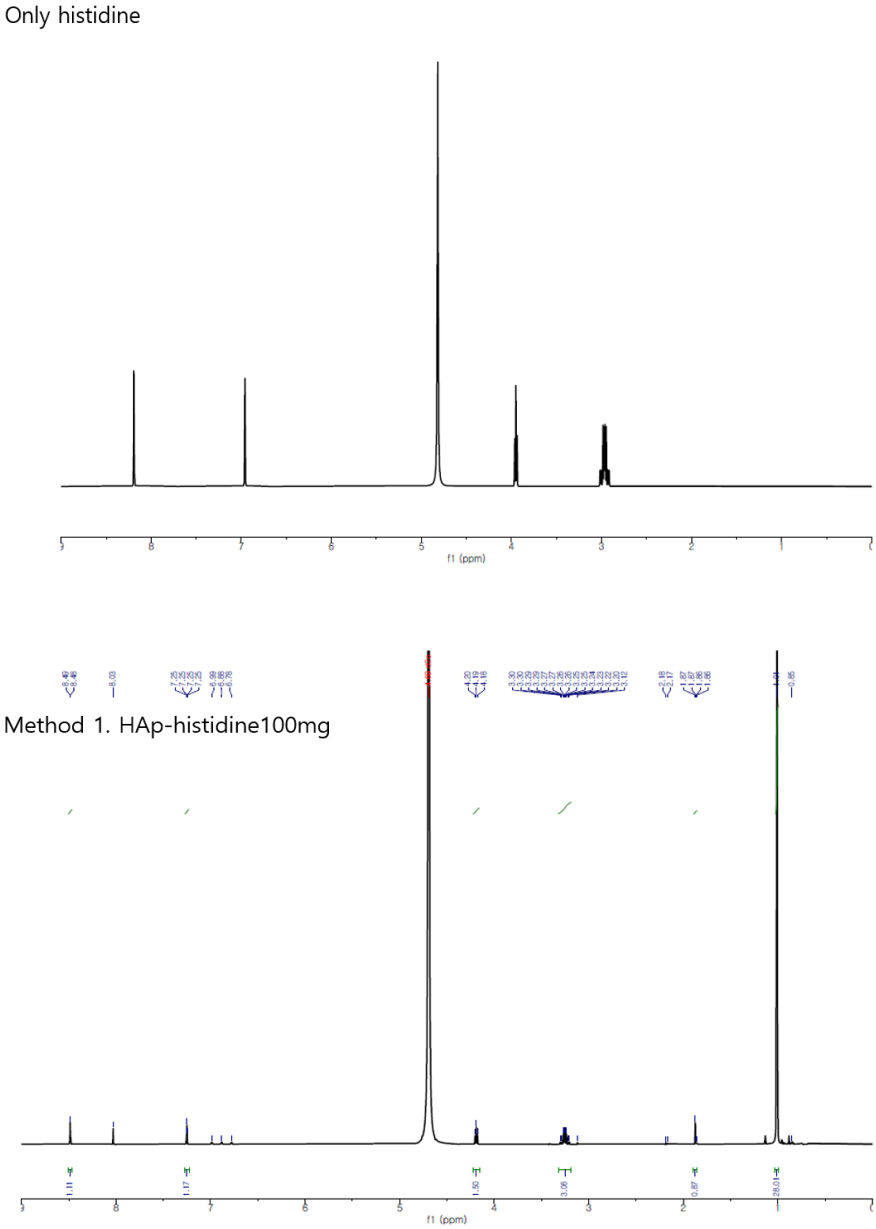


Figure 27. NMR of only histidine and HAp-histidine (method 1).

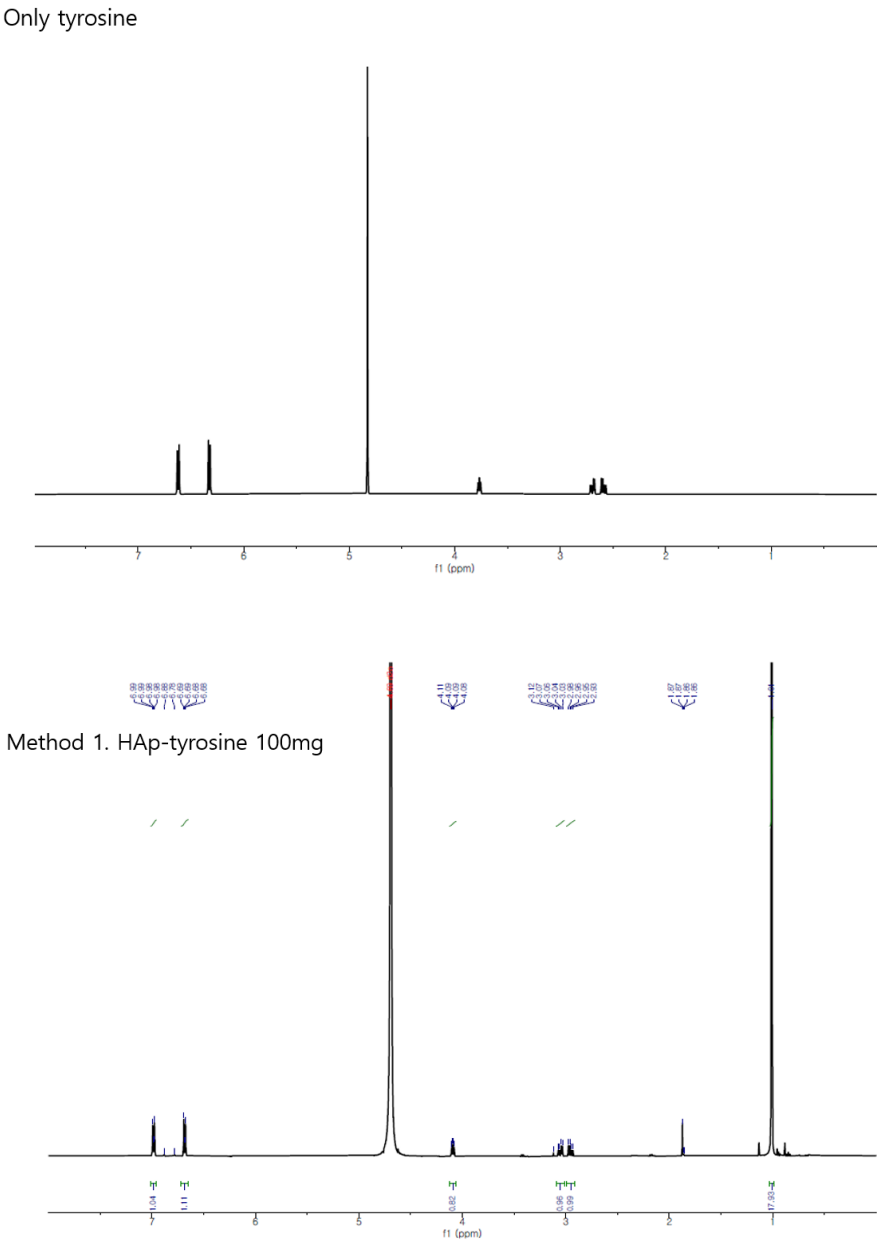
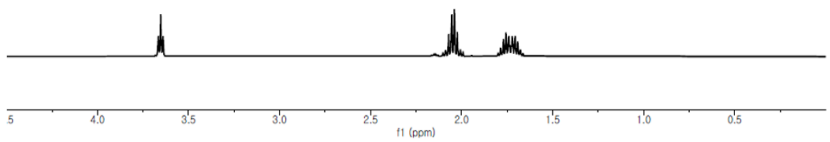


Figure 28. NMR of only tyrosine and HAp-tyrosine (method 1).

Only glutamine



Method 1. HAp-glutamine 100mg

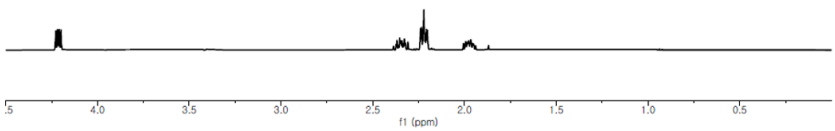
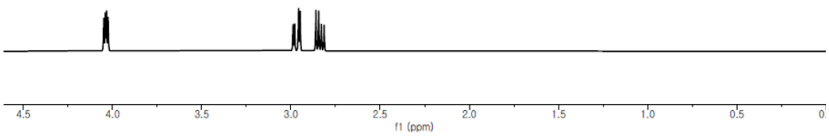


Figure 29. NMR of only glutamine and HAp-glutamine (method 1).

Only cystine



Method 1. HAp-cystine 100mg

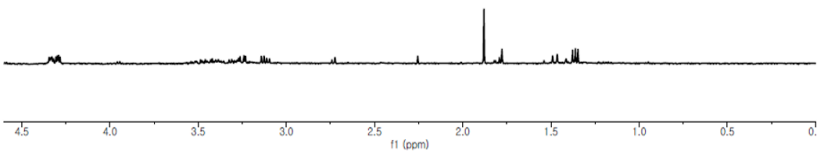
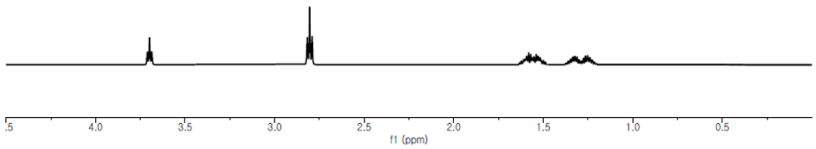


Figure 30. NMR of only cystine and HAp-cystine (method 1).

Only arginine



Method 1. HAp-arginine 100mg

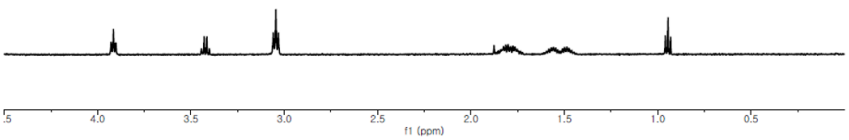


Figure 31. NMR of only arginine and HAp-arginine (method 1).

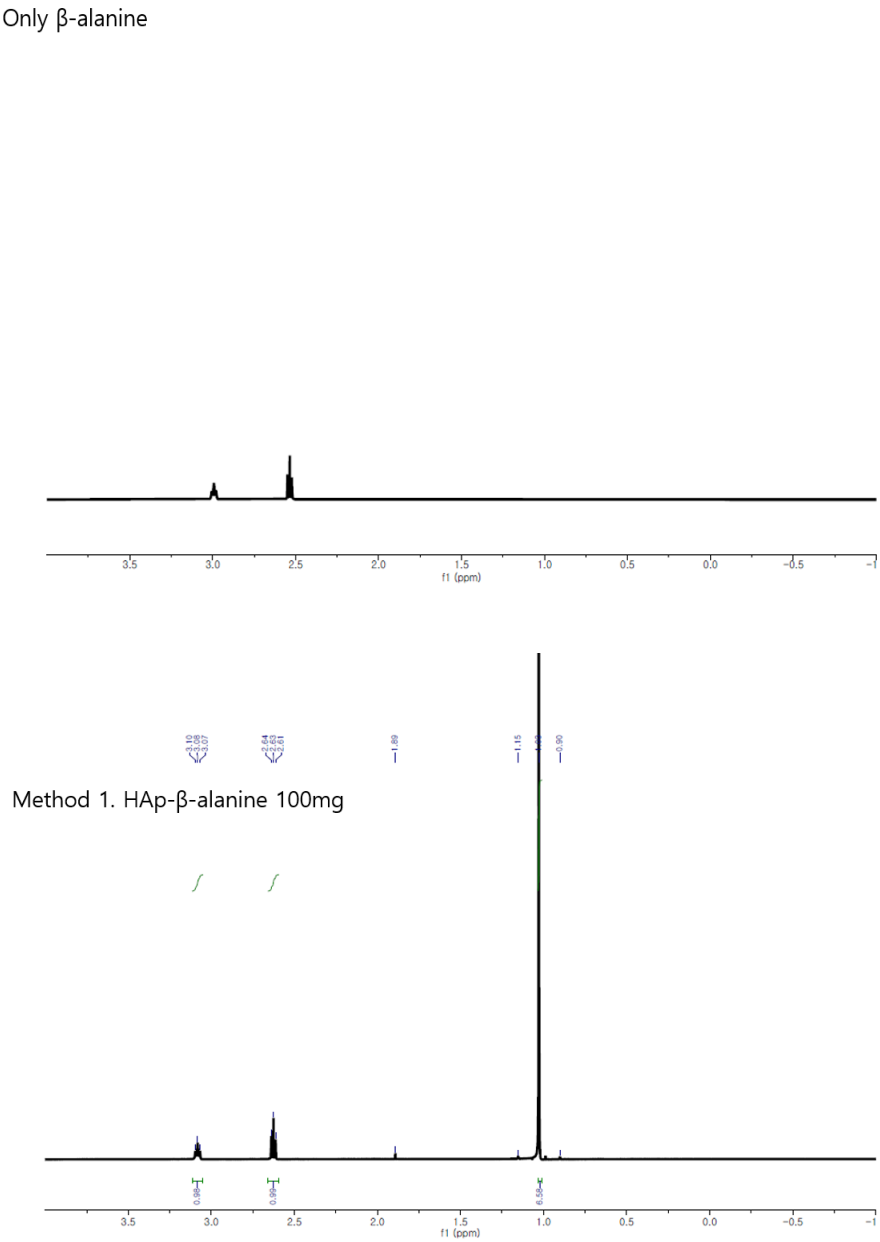


Figure 32. NMR of only β-alanine and HAp-β-alanine (method 1).

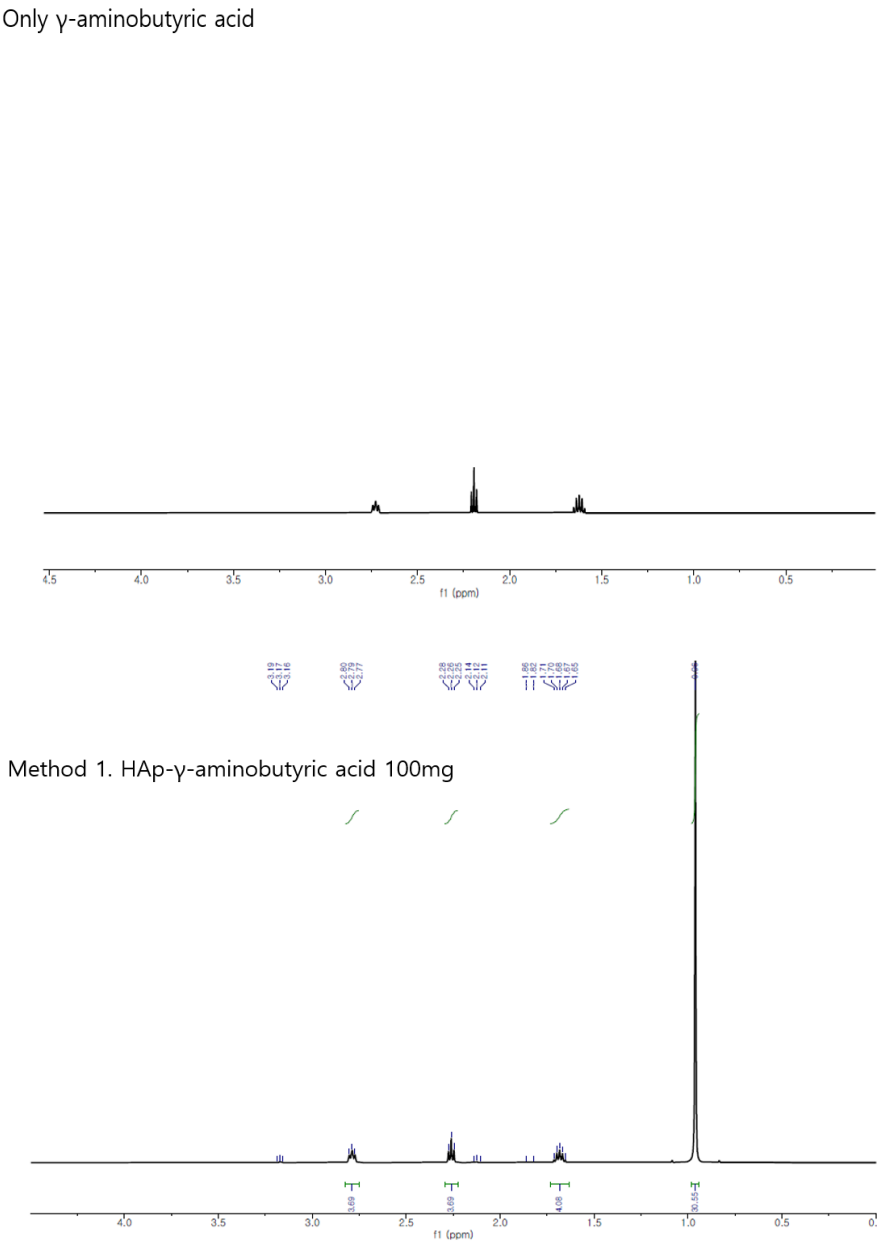


Figure 33. NMR of only γ-aminobutyric acid and HAp-γ-aminobutyric acid (method 1).

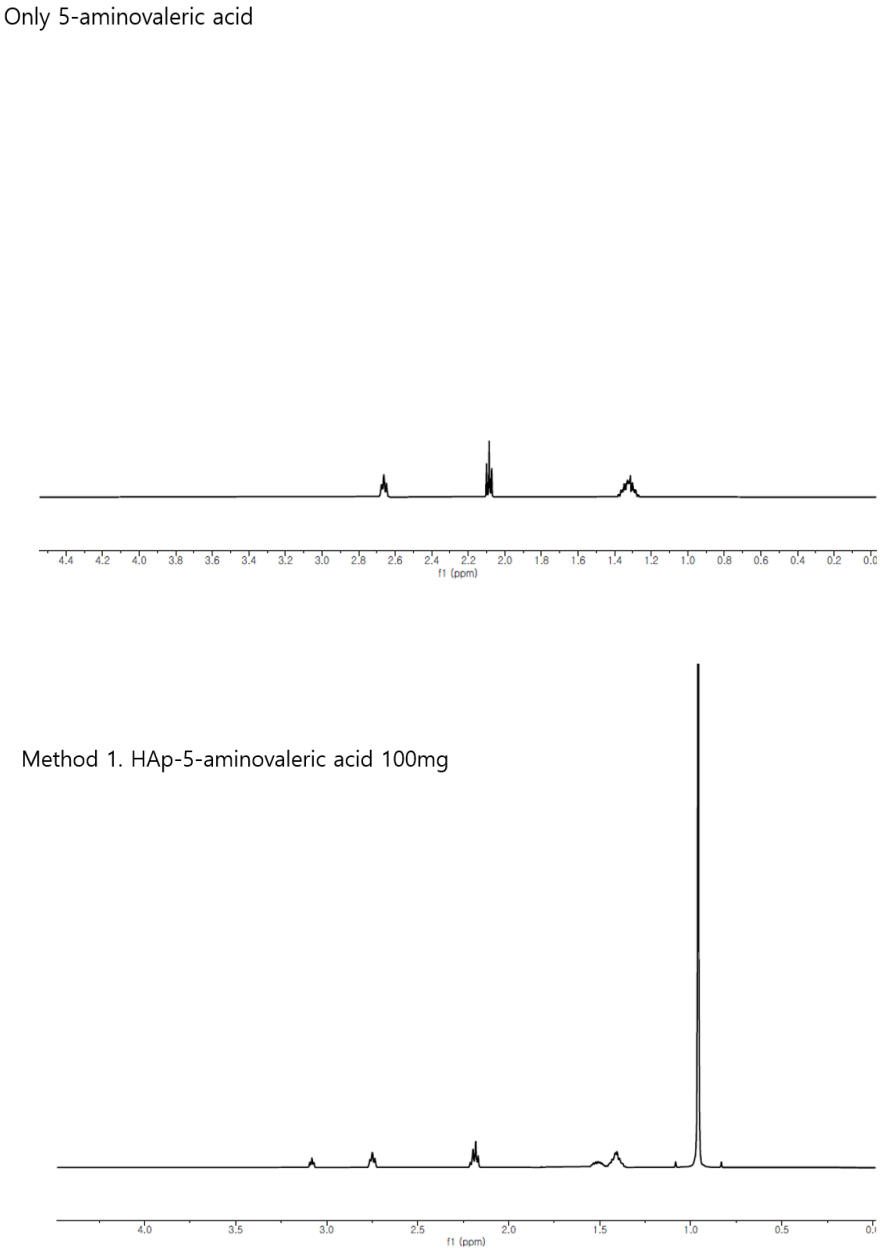
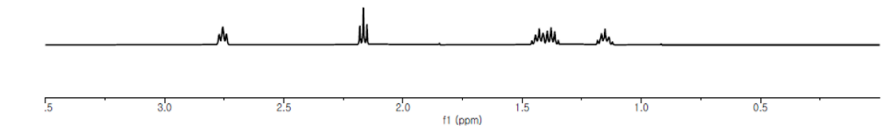


Figure 34. NMR of only 5-aminovaleric acid and HAp-5-aminovaleric acid (method 1).

Only 6-aminocaproic acid



Method 1. HAp-6-aminocaproic acid 100mg

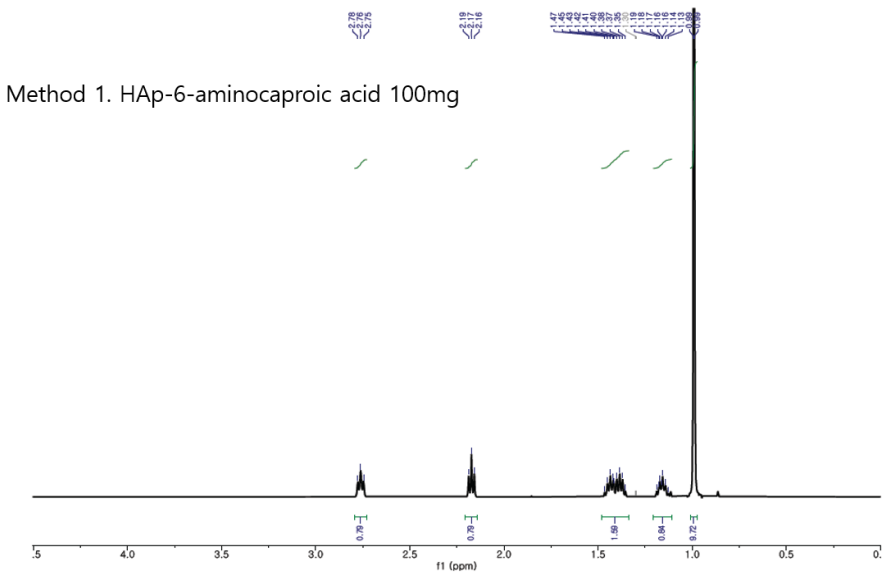


Figure 35. NMR of only 6-aminocaproic acid and HAp-6-aminocaproic acid (method 1).

Conclusion and Summary

In this study, a facile synthetic method and a bio-application of semi-nano hydroxyapatite (HAp) were developed. Microwave-assissted synthetic approach efficiently provides well-crystallized HAp for a short period of time. Besides, lyophilization of subtilisin with hydroxyapatite enhanced the activity of subtilisin in organic media by a factor of 1000. This implies that hydroxyapatite is a useful supporting material for subtilization immobilization.

Many researchers have devised many methods to use hydroxyapatite as a substitute for bone. For this, they have also heavily studied the synthetic methods of hydroxyapatite. Most synthetic procedures require a calcination process, which is generally necessary to provide crystallinity, for a long period of time. However, the microwave irradiation method is the simple, but can provide a large energy for a short period of time. Therefore, it can be used as an alternative of the calcination process. In this study, the idea was exploited, and semi-nano-sized hydroxyapatite with rod shape was synthesized by microwave irradiation. Screening the reaction time and the pH of the reaction mixture revealed that pH 10.6 is critical for proper crystallinity. Furthermore, amino-acid-decorated hydroxyapatites were successfully synthesized by the current method. Sixteen natural amino acids (except glutamine, cystine, and arginine) and three unnatural amino acids were decorated on hydroxyapatite. Syntheses of the amino-acid-decorated hydroxyapatite are not feasible by the classic methods involved in the calcination process.

Subtilisin is a useful enzyme in kinetic resolution of chiral *sec*-alcohols. However, the activity of subtilisin significantly decreases in organic solvents. Hence, researchers have tried to increase the subtilisin activity in organic media. For instance, Clark and cowrokers have reported that the lyophilization of subtilisin with KCl noticeably enhances the activity of subtilisin in organic solvents. Presumably, the charged environment created by KCl efficiently interacts with the hydrophilic surface of subtilisin and prevents subtilisin to be aggregated during lyophilization. This implies that nano-sized materials with charged or polar surfaces can be also used for supporting materials in immobilization of subtilisin. Based on the assumption, hydroxyapatite has been chosen for enhancing stability of subtilisin in organic solvents. To bind HAp with subtilisin, lyophilization was performed. Through this experiment, it has been proved that HAp could enhance the activity of subtilisin as much as KCl.

ABSTRACT

Facile synthesis and application of nano-sized hydroxyapatite for immobilization of subtilisin

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Hydroxyapatite is a main component of natural bone systems, and it is eco-friendly and biocompatible. Researchers have utilized and studied in many bio-applications of hydroxyapatite. In addition, the development of the synthetic methods of hydroxyapatite has also been actively studied. Most synthetic methods include a calcination process, which requires a long period of time and large energy consumption. This thesis deals with a synthetic method of hydroxyapatite in a simple way and for a short period of time, and a utilization for improving the reactivity of subtilisin, which possesses low chemo-tolerance, in organic solvents.

First, hydroxyapatite was synthesized by microwave irradiation for 90 min without a calcination process and any template compounds. SEM analyses revealed that the morphology of the synthesized hydroxyapatite is nano-sized rod with about 150 nm in length and about 20 nm in width. XRD and IR analyses exhibited the patterns of the synthesized

hydroxyapatite are identical with those of the commercial hydroxyapatite. In addition, amino-acid decorated hydroxyapatite was synthesized by addition of amino acids during microwave-irradiation synthesis to expand the bio-application of hydroxyapatite. The presence of the amino acid on the surface was confirmed by NMR analysis with a sample of amino acid-hydroxyapatite degraded by strong acid.

Second, hydroxyapatite was used to improve the activity of subtilisin in organic solvents. Subtilisin is commonly used in the kinetic resolution of chiral sec-alcohols. However, the activity of subtilisin significantly decreases in organic solvents. Therefore, researchers have attempted to improve the reactivity of subtilisin in organic solvents. In this study, it is hypothesized that the charged surface of hydroxyapatite can construct positive interactions with the polar surface of subtilisin, and thus the positive interactions may stabilize subtilisin in organic solvents. As expected, the reactivity of subtilisin lyophilized with hydroxyapatite exhibited a dramatic improvement up to 1000 fold in the reaction of 1-phenylethanol with vinyl butyrate. These results indicates that hydroxyapatite can be efficiently used as an immobilization supporting material for subtilisin.