CURRENT SCIENCE AND TECHNOLOGY (CST)
VOL. 02, ISSUE 1, 67 – 73
DOI: https://doi.org/10.15282/cst.v2i1.8862

ORIGINAL ARTICLE

Synthesis and Characterization of Bromelain Enzyme/Poly (Vinyl Alcohol) as Potential Bone Scaffold

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ABSTRACT – 3D bone scaffold is the temporary constructs substrates that enable and activate the bonding and reproduction of osteoinductive cells that lead to the creation of new usable tissues. The aims of the present study are to synthesize and fabricate bromelain/poly (vinyl alcohol) (PVA) porous substrates by using a lyophilization technique and study the properties of the scaffold subsequently. In this study, bromelain (1, 2 and 3 wt.%) and PVA (15 wt.%) solutions were prepared, then mixed overnight to get a homogeneous solution. The solutions were frozen for 24 hours before being freeze-dried under a vacuum for 7 days at -80 °C. Thereafter, the porous substrates were crosslinked by using glutaraldehyde and characterized using X-ray Diffraction (XRD), Differential Scanning Calorimetry (DSC) and Fourier Transform Infrared Spectroscopy (FTIR). According to the XRD findings, PVA/bromelain scaffolds exhibit great degree of crystallinity at 21.11° and 41.05°. The FTIR results show no significant effect on the absorption peak at a varied concentration of bromelain in bromelain/PVA substrates. For the DSC result, the glass transition temperature was found in the range of 65 to 75 °C. For TGA curve, it was obvious that the temperatures of the weight loss regions gradually increased with the bromelain content, indicating that bromelain improves the stability of the bromelain/PVA membranes at high temperatures. Bromelain/PVA showed high porosity (>75%), and water-swelling ratio within 50 to 150 percent after 24-, 48-, and 72-hours immersion periods. This newly developed scaffold composition may serve as a foundation for the growth of bone tissue.

ARTICLE HISTORY
Received: 05th Aug 2022
Revised: 08th Nov 2022
Accepted: 09th Dec 2022

KEYWORDS
Biopolymers,
Bromelain,
PVA

INTRODUCTION

A critical organ, bone serves several crucial roles in human physiology, including protection, movement, and support for other vital organs [1]. Bones come in a range of sizes and forms. It provides a home for bone marrow, whichmakes blood cells, and stores minerals, particularly calcium [2]. The skeleton of the human body is made up of living tissue that takes the shape of a bone. There are three types of bone tissue: subchondral, cancellous, and compact tissue. On the outside of the bones, there is compact tissue, which is harder tissue. The bone regions that are not subject to a lotof mechanical stress are subjected to cancellous tissue, which has tissue that resembles sponges. Meanwhile, the soft tissue bound by cartilage at the ends of bones is referred to as subchondral tissue [3].

Numerous reports of bone fractures and injuries have been reported worldwide due to trauma, accidents, and illness.Osteomyelitis, burns, and tumours may cause significant skeletal trauma, necessitating rehabilitation surgery to restore anatomy and function as close to normal as possible. Delayed healing or non-union of fractures occurs in five per cent of all fractures and 20 per cent of high-impact fractures [1]. The building of bone, which mimics the extracellular matrix, has encouraged the regeneration of new tissue and stimulated the formation of new tissue [4].

Bone regeneration has been given hope thanks to tissue engineering, which combines cells, bioplymeric scaffolds, and bio factors. In the realm of bone tissue engineering, scaffolds are a sign of progress [5]. In particular, the biopolymers scaffolds should have adequate mechanical characteristics to regenerate hard tissues efficiently. To achieve biocompatibility,neovascularization, biodegradability, and durability, an ideal scaffold should be constructed while considering the bone model's original characteristics. Both synthetic and natural polymers have been widely used as scaffold materials to suit these requirements.

In the past, a combination of bromelain, trypsin, and routine led to a significant and equivalent reduction in pain and inflammation [7]. For six weeks of clinical studies, Brien et al. employed bromelain dosages of 540–1890 mg/day to assess the impact of bromelain on osteoarthritis. [8]. Bromelain's safety profiles are on par with, if not better than, standardtherapy, and it has a high level of endurance and tolerance even at low doses.

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The most frequently utilized water-soluble synthetic polymer for biomedical applications is polyvinyl alcohol (PVA). PVA is hardly soluble in ethanol and insoluble in organic solvents. Due to its solubility pattern and simplicity ofdegradability, it is also referred to as a "green polymer." PVA has a wide range of applications due to its compatibility withvarious polymers and ease of blending with various natural materials. Numerous studies have demonstrated that adding natural fiber and fillers to PVA enhances its mechanical qualities without affecting its capacity to degrade. PVA hydrogelscan be produced by either physical cross-linking through repeated freezing-thawing cycles or chemical cross-linking through glutaraldehyde, etc. [9].

The scaffold is made using a variety of fabrication techniques, such as salt leaching, electrospinning, gas forming,and freeze-drying. One of the simplest methods for creating porous biopolymers scaffolds is freeze drying, which allows for precise control over the scaffold's pore structure. The porosity of freeze-dried biopolymers scaffolds can reach 90% and are exceedingly linked. This procedure adds water to an organic solution containing the desired polymer. The slurryis then added to a mold and quickly frozen using liquid nitrogen. After that, the pressure is lowered to a few millibars, enabling the sublimation of both water and the organic solvent [10].

Through the literature, the present study aims to synthesize and fabricated bromelain/PVA solutions using freeze-drying techniques. The properties of the bromelain/PVA as a potential bone scaffold were obtained and analyzed with X-ray Powder Diffraction (XRD), Thermogravimetric Analyzers (TGA), Differential Scanning Calorimetry (DSC) and FourierTransform Infrared Spectroscopy (FTIR).

EXPERIMENTAL SECTION

Material

The PVA (M_w = 90,000) and bromelain enzyme solution were purchased from Sigma Aldrich, USA. All the purchased chemicals were used without any further purification process. All solutions were prepared by using Millipore water.

Synthesis of Bromelain/PVA scaffolds

Bromelain enzyme solution at varying concentrations of 1, 2, and 3 wt.% was prepared by dissolving in deionizedwater for 12 h. The 15 % PVA was prepared by adding 30 g of PVA granules in 200 ml of deionized water at 175 ℃. Then, 50 ml of the bromelain solution and 50 ml PVA were mixed and stirred overnight to get a homogeneous solution. Then, the solution was divided and poured into three falcon tubes with 40 ml each. All the solutions were frozen at -80
℃ for 12 h followed by freeze-drying under a vacuum at -80 ℃ for 7 days. Next, three frozen sample is crosslinked by immersing them into the mix of 45 ml of acetone solution, 4.5 ml of glutaraldehyde solution, and 7 drops of phosphoric acid, respectively.

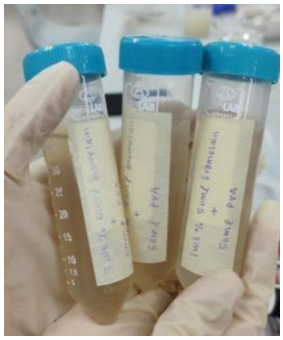

Figure 1. Prepared bromelain/PVA solution

Characterization of bromelain/PVA porous biopolymers.

The crystal structure of the biopolymers is characterized by X-ray Diffractometer (Rigaku Miniflex II) with CuKα(λ=1.5406Å) radiation which is generated at 30 kV and 15 mA. The XRD patterns of the biopolymers are recorded from10° to 80° (2θ)with 0.02° step width. The thermal properties of the bromelain/PVA and biopolymers can be analyzed. The behavior of the samples with temperature was studied using NETZSCH DSC 214 in a nitrogen atmosphere. DSC curves were taken in the range 25 – 400 °C at a heating rate of 10 °C/min. The IR spectra of biopolymers are recorded using a ATR-FTIR (Perkin Elmer, USA) spectrometer. The spectrometer

sends infrared light beams at the sample and detects how much of the beam and at what frequencies thesample absorbs it (Mathias, 2015). The IR spectra were recordedin the transmittance mode using 16 scans over the range500–4000 cm^{-1}. A thermogravimetric analyzer (SDT Q600; TA Instruments, South Korea) was used to characterize the thermal analysis of the biopolymers. The sample weighted at approximately 50 mg. The TGA was conducted until 900 ℃ heating temperature at heating rate of 10 ℃/min under 100ml/min flow rate of nitrogen atmosphere. The liquid displacement method is used to measure the porosity of biopolymers.Firstly, the weight of dried biopolymers and the volume of water are determined. Then, the biopolymers are immersed inthe water for 2 days until complete saturation takesplace and measured the final weight of the biopolymers. As shown inEquation (1), The porosity of the biopolymers can be determined by using the following equation:

$$\text{Porosity } (\%) = \frac{V_1 - V_3}{V_2 - V_3} \times 100 \quad (1)$$

where V_1 is the scaffold immersed in water for 10 min, V_2 is the sum of the weights of the submerged scaffold and water,and V_3 is the water's weight after the scaffold's removal [11].

Lastly, dried biopolymers are weighted and then are immersed in distilled water. The weight of swollen biopolymers is measured at different time period by removed swollen biopolymers from the water and blotted with tissuepaper before being weighted. As shown in Equation (2), the swelling ratio of the biopolymers are calculated by the following equation:

$$\text{Swelling ratio } (\%) = \frac{w_f - w_i}{w_i} \times 100 \quad (2)$$

Where w_i and w_f are the weight of biopolymers before and after socking in the distilled water respectively [13]

RESULT AND DISCUSSION

X-ray diffraction (XRD) study

Figure 2 shows the XRD results of bromelain /PVA scaffold at varied bromelain concentrations. Overall, two Bragg's reflection peaks for the bromelain/PVA solution at 2θ = 21.11° and 41.05°. The previous report by Menazea et. Alshows that PVA exhibits a diffraction peak at 2θ = 19.7 º and 40.4º because of the semi-crystalline PVA structure that is assisted by the intermolecular and intramolecular hydrogen bonds [14]. When PVA interacts with bromelain, the intermolecular connection of PVA chains increases, increasing the degree of PVA-crystalline. The Bromelain/PVA samplesimplied a rise in broadness and peak intensity had been found. This exposes the new material's semi-crystalline character, indicating a higher degree of crystallinity[15].

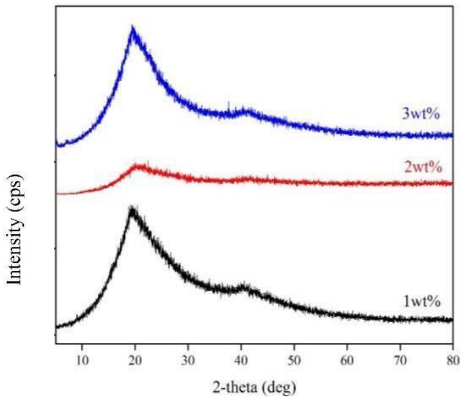

Figure 2. XRD patterns of bromelain/PVA biopolymers at 1, 2 and 3wt.% of bromelain content.

Differential scanning calorimetry (DSC) study

Figure 3 shows the thermograms of bromelain/PVA biopolymers at 1, 2 and 3 wt.% of bromelain content. DSC measurements were used to investigate the thermal characteristics and miscibility of the biopolymers. Under a nitrogen environment, all samples were heated from ambient temperature to 400 °C at a rate of 10 °C min^{-1}.

Knowing that the semi-crystalline PVA polymer has a glass transition temperature of 70 °C [16]. In this study, PVA proves its miscibility with bromelain resulting endothermic peak of the glass transition temperature, T_g being found at 69.7 °C, 71.4 °C, and 73.5°C for 1, 2, and 3 wt.% biopolymers, respectively. The temperature was increased with the bromelain content indicating hydrogen bonding formation between hydroxyl groups of PVA and carbonyl groups of bromelains. Meanwhile, the melting temperature, T_m was found at 161.6 °C, 163.4 °C, and 168 °C for 1, 2, and 3 wt.%, respectively confirming the incorporation of bromelain in PVA biopolymers. Slight exothermic peaks that appear at approximately 180 °C could also be found in all profiles of bromelain/PBA biopolymers.

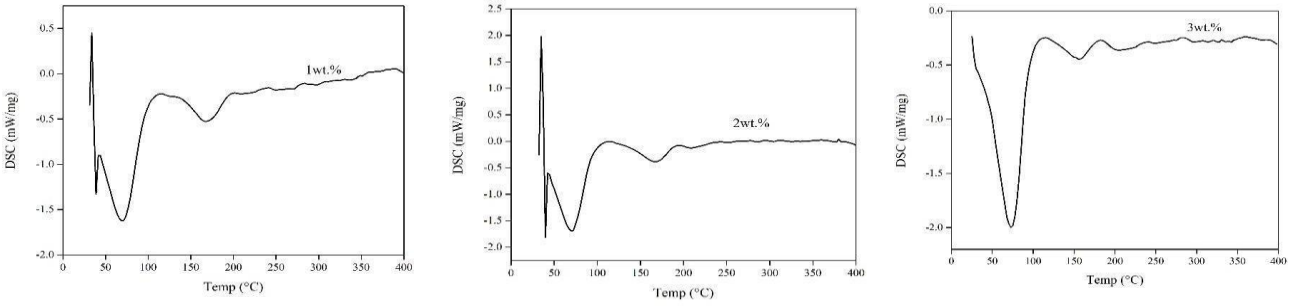

Figure 3. DSC of bromelain/PVA biopolymers at 1, 2 and 3 wt.% of bromelain content..

Fourier transform infrared spectrometer (FTIR) study

Figure 4 shows FTIR spectra of bromelain/PVA biopolymers at 1 ,2 and 3 wt%. Previous study by our groups shows that the FTIR spectra for pure PVA show the functional groups of O-H, C-H symmetric and asymmetric stretching that are assigned to the absorption peak of 3339cm^{-1}, 2919 cm^{-1}and 2854 cm^{-1}, respectively [16]. Meanwhile, the C = O stretching vibration is assigned to the 1719 cm^{-1} bands. The peaks at 1430 cm^{-1}and 1374 cm^{-1} are due to the vibration of C-H bending and C-H wagging. The C–O stretching vibrations band is shown at 1088 cm^{-1} and 1021 cm^{-1} [9]. From Figure3, it was found that the bromelain/PVA has similar functional groups as PVA but slightly different on the peak intensity due to its intermolecular hydrogen bonding between PBA and bromelain. The O-H and C-H symmetric stretching are assigned to the bands at 3443.23 cm^{-1}, 2937 cm^{-1}and for the C-H asymmetric the bands at range 2845-2859 cm^{-1}, respectively. Meanwhile, C = O and C -N stretching vibrations are assigned to the 1712 $cm^{-1}$and 1643 cm^{-1} bands, respectively. The C-N IR spectra were attributed to the stretch vibration of monoalkyl guanidium of pure bromelain. The increasing intensity of C-H bending and C-H wagging bands at 14420 cm^{-1} and 1383 cm^{-1} indicates a stronger hydrogen bond between the polymers.

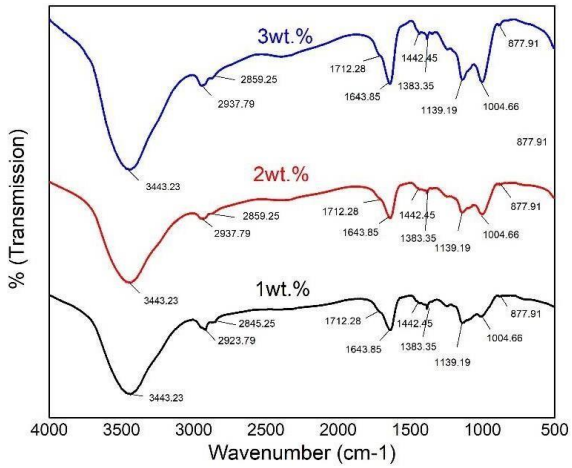

Figure 4. FTIR Spectra of bromelain/PVA biopolymers at 1 ,2 and 3 wt.% of bromelain content.

Thermogravimetric Analysis (TGA)

Figure 5 shows the TGA results for crosslinked biopolymers with different concentrations of bromelain. TGA curve of PVA exhibits three weight loss regions, which are similar to a previous study. The first region between 50 and 200 ◦C can be attributed to the loss of the absorbed water molecules, while the small tangent loss between 200 and 340 ◦C is due to the loss of water bound in the polymer matrix. The third region between 340 and 450 ◦C is associated with the decomposition and carbonization of the polymer. The TGA curves of the (1,2 and 3 wt.%) of bromelain/PVA membranes also exhibited three regions. The firstregion is located between 50 and 200 ◦C and is attributed to water loss.From 200 to 400 ◦C it was due to the decompositionof sulfonic acid groups. The third weight loss region around 400 and500 ◦C was due to the decomposition of the chain of thePVA. According to the TGA curves, it was obvious that the temperatures of the weight loss regions gradually increased with the bromelain content, indicating that bromelain improves the stability of the bromelain/PVA membranes at high temperatures.

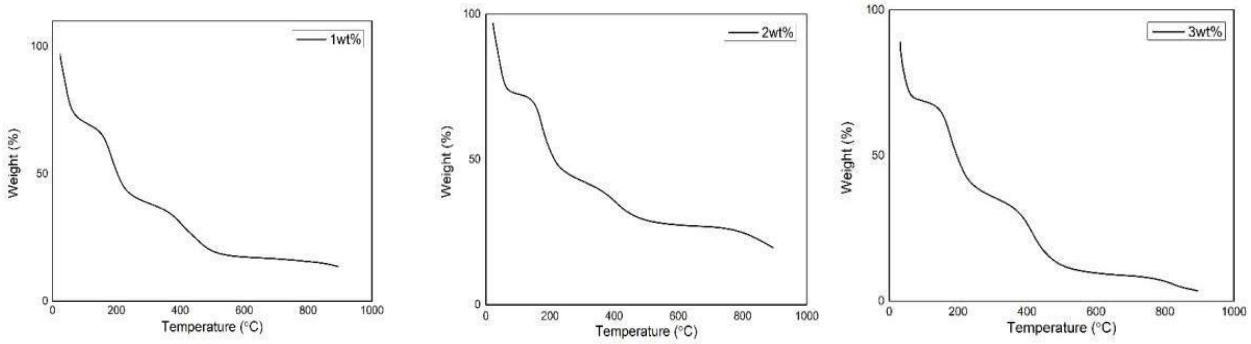

Figure 5. TGA of Bromelain/PVA biopolymers at 1, 2 and 3 wt.% of bromelain content.

Porosity Test

Table 1. Porosity of Bromelain/PVA biopolymers.

Bromelain Concentration	Porosity (%)
1wt%	100.5254
2wt%	99.5931
3wt%	104.3541

Table 1 shows the porosity of bromelain/PVA biopolymers. A scaffold's percentage porosity and pore size play an important role in transporting nutrients and oxygen from the extracellular matrix to the inner surface. Bromelain/PVAscaffold porosities for 1, 2, and 3 wt.% are 100.5254%, 99.5931%, and 104.3541%, respectively. A similar tendency has been seen in the literature, where biopolymers with high porosities (>75%) may accommodate a large numberof cells, allowing in vitro cell adhesion and growth[12]. The findings revealed that the scaffold had adequate porosity topromote bone cell development. In general, the best porous biopolymers for tissue engineering have a proper aperture, a homogenous microstructure, and a high porosity. Higher porosity biopolymers give more excellent room and nourishmentto cells and tissue than lower porosity biopolymers.

Swelling Test

Table 2. Swelling ratio of Bromelain/PVA biopolymers

Bromelain Concentration	Swelling ratio (%)		
	Immersion Time (h)		
	24h	48h	72h
1wt.%	52.84	54.87	57.77
2 wt.%	105.56	111.09	114.67
3 wt.%	123.62	128.74	132.68

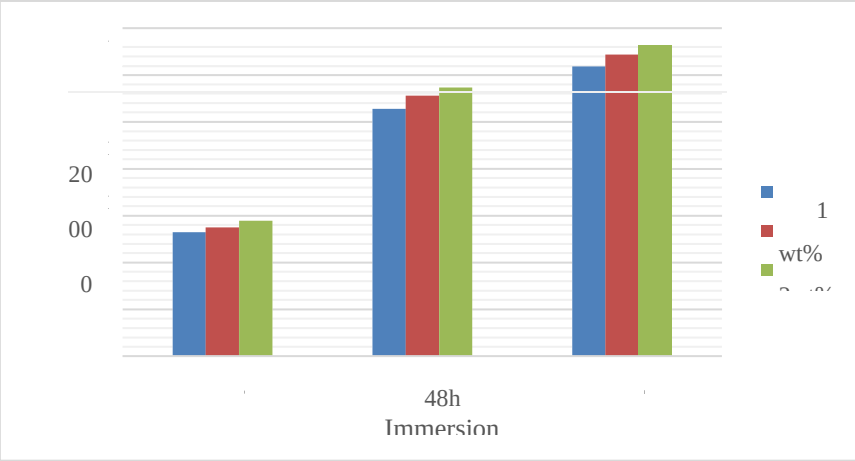

Figure 6. Swelling behaviour of bromelain/PVA biopolymers

The results of the produced scaffold's water absorption are displayed in Figure 6. It can be seen that after 24, 48 and 72 hours, the percentage of water absorption grew steadily from 50 to 150%. The swelling ability of hydrogels isn't just determined by porosity; the micro-architecture of the porous network can also influence the water absorption capacityof hydrogels. [12]. The swelling ability of biopolymers allows cells to infiltrate the biopolymers in a three-dimensional manner. Swelling also increases pore size and overall porosity, optimizing the biopolymers' interior surface area.

CONCLUSION

In conclusion, the bromelain/PVA scaffold was synthesized through a freeze-drying technique. In this research, The XRDresult of PVA interacting with bromelain indicates a higher degree of crystallinity. This reveals the semi-crystalline natureof the new substance. The crystalline nature of PVA was detected to form strong interaction between PVA through intermolecular hydrogen bonding. FTIR results show the functional groups such as O-H, C-H symmetric, C-H asymmetric, C = O, C = C, C-H bending, C-H wagging, C–O stretching vibrations. The glass transition temperature for the DSC result was somewhat different in the region of 65 °C to 75 °C which is lower than pure PVA's T_g value. For the TGA curve, it was obvious that the temperatures of the weight loss regions gradually increased with the bromelain content, indicating that bromelain improves the stability of the bromelain/PVA membranes at high temperatures. The higher the glass transition temperature, the higher the thermal stability due to the increased bromelain. The porosity results show that bromelain/PVA has a high porosity (>75%), which would stimulate bone cell development. Finally, the swelling resulted after 24, 48, and 72 hours, and the percentage of water absorption grew steadily from 50 to 150%. The higher the bromelain concentration, the higher the value of porosity and swelling abilities properties. Therefore, based on this the preliminary results of the properties of bromelain/PVA biopolymers, this finding could be a reference to explore its usage in bone tissue applications.

ACKNOWLEDGEMENT

The authors would like to thank the Universiti Malaysia Pahang for laboratory facilities as well as additional financial supportunder Internal Research grant RDU210331 and Postgraduate Research Scheme grant no. PGRS2003110.

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