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Cross-Linking of Thermally Hydrolyzed Specified Risk Materials with Epoxidized Poly (Vinyl Alcohol) for Tackifier Applications

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Abstract: Prions have been identified as the infection source for bovine spongiform encephalopathy or 'mad cow disease'. Safety concerns relating to this disease have led to strict feed regulations for specified risk materials (SRMs) in North America, which are characterized as the tissues in cattle where prions are likely to concentrate. As one of the approved SRM disposal methods, thermal hydrolysis converts proteinaceous materials, including prions into non-infective peptides, which have been examined for incorporation into a variety of value-added applications. Here, we describe the bio-conversion of SRM-derived peptides into tackifiers for hydro-mulch applications. Tackifiers are employed in erosion control strategies and help to bind seed and mulch to eroded areas to promote the restoration of vegetation. In this study, epoxidized poly (vinyl alcohol) (PVA) was synthesized and employed for cross-linking of SRM-derived peptides. The reaction conditions and the molar ratio of the reagents applied for the cross-linking reaction were shown to have significant effects on cross-linking behaviour. Furthermore, SRM-derived peptides that were modified with epoxidized PVA displayed viscosity, binding, and moisture maintaining capacity that were comparable to commercially available tackifiers. Hence, this research further strengthens the argument for using SRM-derived peptides as feedstock for sustainable tackifiers development.

Keywords: peptides cross-linking; epoxidized PVA; SRM-derived peptides; tackifiers

1. Introduction

Prions are believed to be the source of infection of bovine spongiform encephalopathy (BSE), a transmittable neurodegenerative disease found in cattle. To defend against potential prion infection, the cattle tissues that have a substantially higher risk of prions aggregation, characterized as specified risk materials (SRM), must be separated and disposed of using specific protocols. These tissues, including the brain, skull, eyes, trigeminal ganglia, spinal cord, vertebral column (with some exclusions), and dorsal root ganglia (DRG) of cattle 30 months of age and older, as well as the tonsils and distal ileum of the small intestine of all cattle [1,2], have been limited and even banned from use as animal feed, pet food, and fertilizers since the 1980s [3]. The prohibition of SRM usage resulted in economic losses for the rendering and processing industries since they were now forced to dispose of SRM via incineration or landfilling [4]. For instance, over 300,000 tonnes of SRM are segregated and disposed of annually in Canada [4], resulting in over 22.5M CAD$ of additional costs for the rendering industries, based on a disposal cost of 75–200 CAD$/tonne per year [5]. To reduce animal by-product waste from the livestock industry, many studies have examined the incorporation of SRM into value-added products.

The potential of SRM biomass is indicated by the predominant protein content (48.7%) [5], which can be converted into products of value and sustainability. Owing to the extreme resistance of

prion aggregates with regards to conventional decontamination techniques [1], various protocols were proposed by the Canadian Food Inspection Agency (CFIA) and the Food and Drug Administration of the United States (FDA) [6]. Among those protocols, thermal hydrolysis has been drawing more and more attention [4,7]. One of the main reasons is that this hydrolysis protocol has been demonstrated to completely destroy any prions present [4,8], while also facilitating the recovery of peptides or protein fragments that can potentially be utilized in various bio-conversion processes for generation of biodegradable and/or renewable industrial products, such as tackifiers.

Tackifiers, which are mostly composed of hydrophilic polymers, are often used as an additive in hydro-mulching processes involved in erosion control. Hydro-mulch helps to promote the restoration of vegetation and maintain surface water in drier environments. Within hydro-mulch, tackifiers are expected to bind seed with mulch fibres and soils to fix the seeds to the targeted land [7,9]. Currently, the hydro-mulch industry typically adds synthetic polymers, such as polyacrylamide (PAM), as tackifiers. PAM has great binding strength; however, it is non-biodegradable and is relatively expensive (US$8.12/kg) [10]. Moreover, guar gum, psyllium, and corn starch, three types of relatively new bio-degradable tackifiers obtained from plants, can substitute for PAM, but are currently limited in their applications due to their lower binding performance and increasing prices, which are as much as US$6.00/kg (guar), US$1.67/kg (psyllium), and US$1.44/kg (corn starch) [11–14]. Therefore, development of a cost-effective, biodegradable tackifier with comparable qualities to commercial tackifiers would be of significant value to the erosion control industry.

In a previous study from our group, we showed that SRM-derived peptides, as well as peptides cross-linked with glutaraldehyde, could be employed as tackifiers [7]. The binding strength of these SRM-derived tackifiers demonstrated similar binding capacity as commercial tackifiers (i.e., guar gum). However, by using other cross-linkers, such as epoxide cross-linkers, it may be possible to develop a novel SRM-based tackifier with enhanced binding strength that is more comparable to PAM.

Epoxides with multiple epoxy groups can be used as cross-linkers to react with the functional groups present in peptides, including amines, hydroxyl groups, carboxylic groups, and sulfhydryl groups (Figure 1) [15]. Based on a study by Shechter et al. that examined reactions between glycidyl ether and amines in different conditions, at ambient temperature, the reaction of epoxy groups with amines was shown to proceed faster than reactions with other functional groups such as carboxylic or hydroxyl groups [16]. Long chain epoxides can also be applied as a cross-linker. This can be done by grafting epoxy groups to a polymer chain (such as poly (ethylene glycol) (PEG), PVA, etc.), which could facilitate cross-linking with functional groups such as hydroxyl groups, carboxylic groups, and amines. For instance, the epoxidation of PEG by epichlorohydrin at different molar ratios was successfully achieved by Motawie et al. and Mingzhong et al., and the products were employed for the cross-linking of bisphenol and silk fibre, respectively [17,18]. Increases in water resistance and adhesion of the products were observed in their studies, which indicated the possible change in properties offered by epoxide cross-linking [17,18]. It is worth noting that PVA, a hydrophilic polymer that adopts a globular structure in solution [19], was studied by Iulian et al. for the synthesis of super absorbent hydrogels through cross-linking with epichlorohydrin [20]. In this report, epoxy groups were grafted to the PVA chain, enabling PVA to be used as an epoxide cross-linker. However, thus far, there have been no reports on subsequent cross-linking between epoxidized PVA and peptides/proteins.

In this report, epoxidized PVA, an epoxide product of the reaction between PVA and epichlorohydrin, was first synthesized and the reaction varied to achieve high levels of epoxy content. The epoxidized PVA was then studied as a cross-linker for SRM-derived peptides under various conditions (i.e., temperature, molar ratio, and reaction time). Finally, the cross-linked products obtained from cross-linking reactions were evaluated as tackifiers using previously established methods. In summary, this study further strengthens the potential of using by-product waste from the livestock industry as a feedstock for the development of tackifiers. These tackifiers would have a competitive advantage over current commercial tackifiers since they are prepared from negative-value materials, and are also biodegradable, nutrient-rich, of high binding strength, and have water-holding capacity.

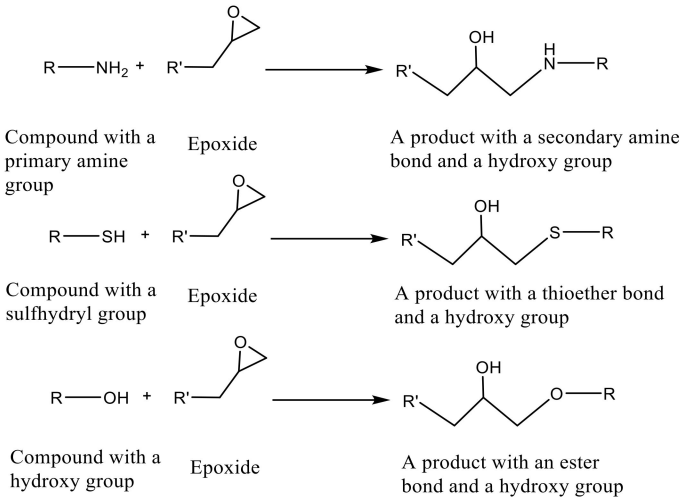


Figure 1. The reactions of an epoxy group with a primary amine group, a sulfhydryl group, and a hydroxyl group, respectively.

2. Materials and Methods

2.1. Materials

SRM was kindly provided by a rendering facility in Western Canada. L-Leucine (reagent grade, ≥98%), (±)-epichlorohydrin (purum, ≥99%), hydrochloric acid (certified ACS, 37%) and poly (vinyl alcohol) (MW 13000-23000, 87-89% hydrolyzed) were procured from Sigma-Aldrich (St. Louis, MO, USA). 0.1 M standard sodium hydroxide solution (0.0995 to 0.1005 mol/L at 20 °C), 0.1 M standard hydrochloric acid solution (0.0999 to 0.1001 mol/L at 20 °C), sodium hydroxide (certified ACS, 98.8%), hexane (certified ACS), acetone (certified ACS), cresol red (pure, indicator grade), and thymol blue (pure, indicator grade) were purchased from Fisher Scientific (Waltham, MA, USA).

2.1.1. SRM Hydrolysis and Recovery

The thermal hydrolysis of SRM was conducted in a dedicated 5.5-L stainless steel pressure vessel (Parr 4582, Parr Instrument Company, Moline, IL, USA). The vessel was equipped with a Parr reactor controller (Parr 4848, Parr Instrument Company, Moline, MI, USA) to monitor the temperature and the pressure of hydrolysis [6]. By following the optimized protocol developed by Mekonnen et al. [4], a typical SRM hydrolysis and peptides recovery procedure was followed, starting with mixing of SRM (1.0 kg) with Milli-Q water (1.0 L) in the pressure vessel. The mixture was then processed at 180 °C and ≥174 psi pressure for 40 min to complete the hydrolysis. After the hydrolysis, 9.0 L of Milli-Q water was added to the hydrolysate to facilitate dilution. The resulting suspension was then subjected to centrifugation (Avanti J-26 XP high-performance centrifuge, Beckman Coulter, Mississauga, Canada) at 7000 × g for 40 min, followed by filtration of the supernatant using Whatman no. 4 filter paper (20–25 µM pore size). The filtrate was later washed with hexane (1:1 v/v ratio, three times) and subjected to lyophilisation for the recovery of tan-coloured powders, termed SRM-derived peptides. According to our previous study [7,21], SRM-derived peptides were characterized as being comprised of 0.59 ± 0.05 mmol/g of primary amine groups and 1.69 ± 0.06 mmol/g of carboxylic acid groups.

2.1.2. Epoxidation of PVA

In a typical experiment, 1.14 g of PVA was dissolved in 5.0 mL Milli-Q water at 60 °C for about 10 min. Following this, 6.0 mL of epichlorohydrin was added into the pre-cooled PVA reactant at a molar ratio of 1:3 (hydroxyl groups: epoxy groups) (Table 1). Other molar ratios (1:0.5, 1:1, 1:2, and 1:4) were also tested (Table 1). The reaction was carried out in an oil bath at a given temperature for 3–20 h. After the reaction, 1.20 mL of aqueous NaOH solution (0.75–7.00 mol/L) was added to the reaction at a

rate of 200 µL/20 min to facilitate dechlorination. After cooling of the reaction to room temperature, the product solution was diluted with 15.0 mL Milli-Q water followed by a hexane wash (30 mL) to remove excess epichlorohydrin. The hexane-washed solution was later neutralized with 0.10 mol/L HCl, to produce epoxidized PVA.

Table 1. The molar ratio design for epoxidized PVA synthesis.

PVA (g)	No. of Hydroxyl Groups (mmol)	Epichlorohydrin (mL)	No. of Epoxy Groups (mmol)	Molar Ratio of Hydroxyl Groups: Epoxy Groups
1.14	25.8	1.0	12.9	1:0.5
1.14	25.8	2.0	25.8	1:1
1.14	25.8	4.0	51.6	1:2
1.14	25.8	6.0	77.4	1:3
1.14	25.8	8.0	103.1	1:4

2.1.3. Epoxy Content Determination

An adapted method was used for measuring the epoxy content of the epoxidized PVA, which was based on an ultrasonication-assisted determination method developed by Zhipeng et al. (2014) [22]. In brief, the reaction indicator was prepared by mixing 0.1% cresol red solution with 0.1% thymol solution at a volume ratio of 1:3, followed by pH adjustment to 7.0 with 0.01 mol/L sodium hydroxide aqueous solution. To measure epoxide content, epoxidized PVA (synthesized using 1.14 g PVA) was mixed and reacted with 10.0 mL of a hydrochloric acid-acetone solution (1:40 volumetric ratio), followed by ultrasonication (Crest P500 ultrasonic cleaner, Crest Ultrasonics Corp., Ewing, NJ, USA) at 23 °C for 4 min. After this, 4 drops of reaction indicator were added to the solution. Subsequently, the solution was titrated with 0.10 mol/L NaOH standard solution. The blank (control) experiment was also conducted in the same manner, but 25.0 mL Milli-Q water (pH 7.0) were used instead of epoxidized PVA. The epoxy content was calculated using Equation (1) as follows [22]:

$$\textit{Epoxy content}\ (\text{mmol}) = [V_0\ (\text{mL}) - V\ (\text{mL})] \times C_{NaOH}\ (\text{mol/L}) \quad (1)$$

where V_0 is the volume of NaOH standard aqueous solution consumed in the blank (control) experiment with Milli-Q water, V is the volume of NaOH standard aqueous solution consumed by epoxidized PVA reaction, and C_{NaOH} is the concentration of NaOH used for the titration.

2.1.4. Cross-Linking of SRM-Derived Peptides with Epoxidized PVA

For the cross-linking reactions, SRM-derived peptides in different amounts (i.e., 8.00, 4.00, 2.00, and 1.50 g) were added to the entire epoxidized PVA reactant (15 mL) obtained from the epoxidation of 1.14 g PVA. After mixing, the pH of the reaction mixture was adjusted to 9.0 using 3.00 mol/L NaOH before the reaction was carried out at a particular temperature (23–80 °C) for 3–20 h in an oil bath. Subsequently, the reaction was neutralized with 0.10 mol/L NaOH, followed by sample collection through lyophilization. The products were termed as PVA-EPC-peptides (PEP). The various reaction conditions examined for cross-linking of SRM-derived peptides with epoxidized PVA are shown in Table 2.

Table 2. Cross-linking reactions performed using epoxidized PVA and SRM-derived peptides.

Epoxidized PVA (mL)	No. of Epoxy Groups (mmol)	Mass of the SRM-Derived Peptides (g)	Molar Ratio of Epoxy Groups: Primary Amino Groups
15	2.33	8.00	0.5:1
15	2.33	4.00	1:1
15	2.33	2.00	2:1
15	2.33	1.30	3:1

2.1.5. Primary Amino Groups Characterization

The TNBSA method, a derivative method developed by Hermanson (San Jose, CA, USA) [23], was applied for the characterization of primary amino group content in the reacted product. L-leucine was used as the standard to establish a calibration line.

2.1.6. Carboxylic Groups Characterization

A pH titration method developed by Claudia et al. [24] was employed to measure the carboxylic acid groups remaining in the cross-linked products. The number of carboxyl groups was calculated by multiplying acid dosage by the acid strength. The residual –COOH (%) can be calculated using Equation (2) as follows:

$$\begin{aligned} Residual-COOH\ (\%) &= \frac{N_{-COOH}\ (\text{mol})}{N'_{-COOH}\ (\text{mol})} \times 100\% = \frac{N_{-COOH}\ (\text{mol})}{m_P\ (g) \times 1.69 \times 10^{-3}\ \left(\frac{\text{mol}}{\text{g}}\right)} \times 100\% \\ &= \frac{C_{HCl}\ (\text{mol/L}) \times V_{HCl}\ (\text{mL}) \times 10^{-3}}{m_P\ (\text{g}) \times 1.69 \times 10^{-3}\ (\text{mol/g})} \times 100\% \end{aligned} \quad (2)$$

where $N_{\text{-}COOH}$ is the number of carboxylic groups quantified in the product solution. $N'_{\text{-}COOH}$ is the number of carboxylic groups in the original SRM-derived peptides, which can be calculated by multiplying the mass of peptides used for the reaction (m_p) by the content of carboxylic groups in SRM-derived peptides as determined previously (1.69 mmol/g) [7]. V_{HCl} is the volume of HCl used for the titration and C_{HCl} is the concentration of HCl.

2.1.7. Compression Strength Analysis

The binding strength of the cross-linked products was assessed by creating pucks with MulchMax 101 wood fibres using a method developed in our previous tackifier study [7]. To make a puck, 0.40 g of the cross-linked product was first added to 20.0 mL Milli-Q water to prepare the tackifier suspension, followed by uniformly mixing the tackifier suspension with 4.00 g of MulchMax 101 wood fibres (Synermulch Erosion Control Products Inc., Calgary, Canada). An aluminium weighing dish (44 mm diameter inner base, Fisher Scientific, Ottawa, Canada) was then used as a mould to store the mixture, and to fix the shape of the mixture via a curing process at 40 °C for 48 h in a drying oven (Isotemp 625G Gravity Oven, Fisher Scientific, Ottawa, Canada). After that, the mixture was cooled at room temperature for 24 h.

To assess binding strength of the tackifier, the pucks made using the cross-linked product were compressed to their breaking point using a *TA.XTPlus* Texture Analyser (Texture Technologies Corp. and Stable Micro Systems Ltd., Hamilton, Canada) at a drop rate of 5 mm/s. The breaking force (N) recorded by the analyser was deemed as the compression strength of the pucks, which was directly related to the binding strength of the cross-linked product.

2.1.8. Thermogravimetric Analysis (TGA)

The thermal stability of the cross-linked products was studied with a Thermal Analysis Instruments Q500 (TA Instrument, New Castle, DE, USA) under a nitrogen atmosphere. Platinum pans were used as sample holders. Samples with a mass of 15–20 mg were inserted into the platinum pans and the

temperature was ramped from 24 °C to 100 °C (held for 10–15 min to facilitate moisture removal), then heated to 350 °C at a heating rate of 5 °C/ min.

2.1.9. Moisture-Holding Capacity

To assess the water-holding capacity of the cross-linked products, a method developed in our previous study was utilized [7]. In a typical measurement, the sample suspension was first prepared by mixing 1.00 g of sample powders with 20.0 mL of Milli-Q water. To reach a homogeneous state, the suspension was then stirred on a magnetic plate stirrer at a rate of 400 rpm for 2 h. After the preparation, the suspension was uniformly spread in a plastic Petri dish (100 mm × 15 mm, Fisherbrand, Ottawa, Canada) followed by an incubation at 23 °C for 60 h in a New Brunswick Innova 4900 Incubator Shaker (New Brunswick Scientific Co. Inc., Enfield, CO, USA). The relative humidity of the incubator was set at 0.0%. Finally, a moisture analyzer (HE53 Halogen, Mettler Toledo, Columbus, OH, USA) was employed to determine the moisture content of the residues collected in the dish, which was recorded as the moisture-holding capacity of the sample.

2.1.10. Aqueous Viscosity Analysis

A Model 900 Viscometer (OFI Testing Equipment Inc., Houston, TE, USA) was used to assess the viscosity of the aqueous suspensions. To prepare the suspension, 0.84 g of samples was added to 175 mL of Milli-Q water followed by vigorous shaking for 5 min. The prepared suspension was then analysed at 20 °C at a shear rate of 300 rpm to determine the viscosity (cP).

2.1.11. Statistical Analysis

The values in this study were statistically analysed via Tukey's HSD (honestly significant difference) method [25]. In a given figure, values that share the same letter are statistically similar at a 95% confidence level.

3. Results and Discussion

3.1. Cross-Linking of SRM-Derived Peptides with Unmodified PVA

Previous work from our group has shown that SRM-derived peptides could be potentially applied in the development of an industrial tackifier. However, compared with commercial tackifiers such as starch and psyllium, both SRM-derived peptides and glutaraldehyde cross-linked peptides (glutaraldehyde-peptides), exhibited lower binding strength and water-holding capacity [7]. To develop a tackifier that is more comparable to starch and psyllium, poly (vinyl alcohol) (PVA), a water-soluble synthetic polymer, was introduced as the cross-linker. The abundant hydroxyl groups in the PVA molecule not only impart promising hydrophilicity, but also make the cross-linking with the amino groups and carboxylic groups of SRM-derived peptides possible for tackifier development. However, the amount of residual carboxylic groups in SRM-derived peptides after cross-linking with PVA was found to be 80.5–89.9% after 12 h at a 1:1 molar ratio (hydroxyl groups in PVA: carboxylic groups in peptides) using different solvents (i.e., water and dimethylformamide) and temperatures (i.e., 23–100 °C for water, 23–150 °C for dimethylformamide) (Figure 2). These results demonstrated a relatively low reactivity between hydroxyl groups of PVA and carboxylic groups of SRM-derived peptides.

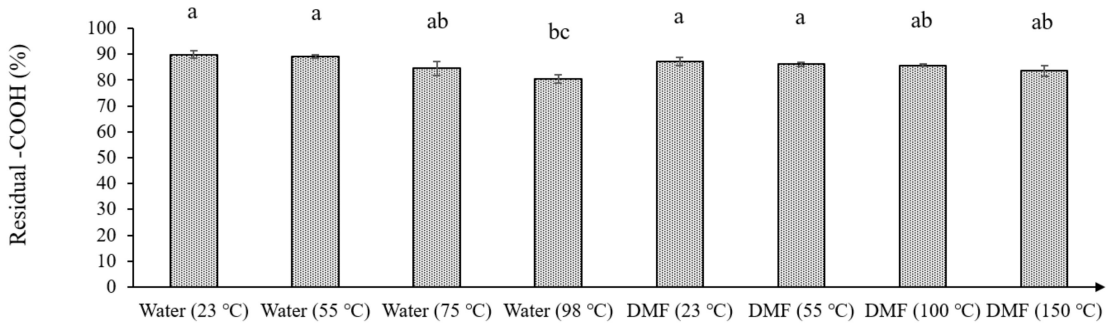


Figure 2. The residual carboxyl groups after reactions of SRM-derived peptides with PVA. Reactions were performed in aqueous (water) or non-aqueous (DMF; dimethylformamide) environments at a variety of temperatures. Values represent the average mean ± standard deviation of triplicate experiments. Values that do not share the same letter indicate that they are significantly different ($p < 0.05$).

3.2. Synthesis and Characterization of Epoxidized PVA

In an attempt to improve the cross-linking reaction, we introduced an epoxide group into PVA, as epoxidized PVA is known to be highly reactive [16]. Epoxidized PVA is a substitutional product of PVA that can be generated via grafting of epoxy groups to its hydroxyl groups. Due to the promising reactivity of epoxy groups with both amino and carboxylic groups of SRM-derived peptides, epoxidized PVA was expected to be more effective for cross-linking of SRM-derived peptides.

According to the mechanism of polymer epoxidation, both poly (ethylene glycol) (PEG) diglycidyl ether and epoxidized PVA can be produced via reaction with epichlorohydrin [19,20,26]. Moreover, compared with PEG diglycidyl ether, PVA possesses a higher amount of hydroxyl groups per molecule that might be useful as cross-linking sites, but can also facilitate self-polymerization (i.e., formation of a PVA hydrogel; Figure 3) during the epoxidation. The self-polymerization of PVA could result in wasted epichlorohydrin and reduce the epoxy content of the epoxidized products. Thus, the synthesis of epoxidized PVA with a high epoxy content is necessary for subsequent cross-linking of SRM-derived peptides. To this end, an extensive study on the reaction conditions (i.e., concentration of NaOH aqueous solution, temperature, and reaction time) was conducted to maximize production of the desired epoxidized product.

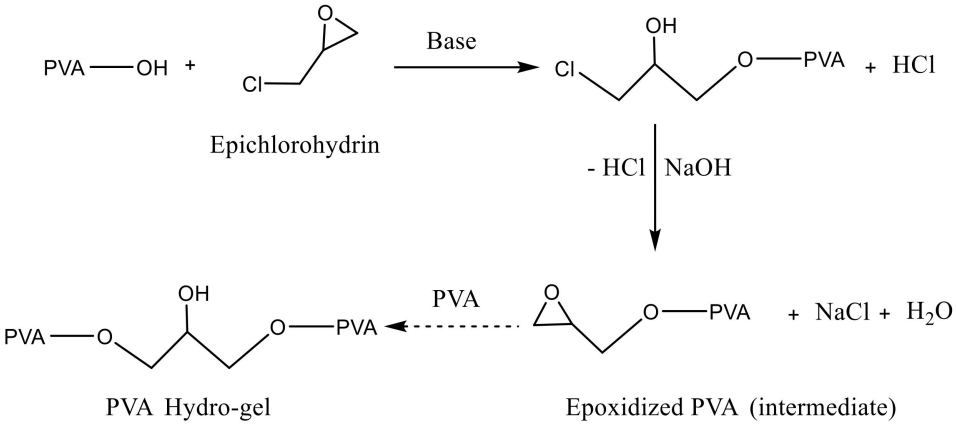
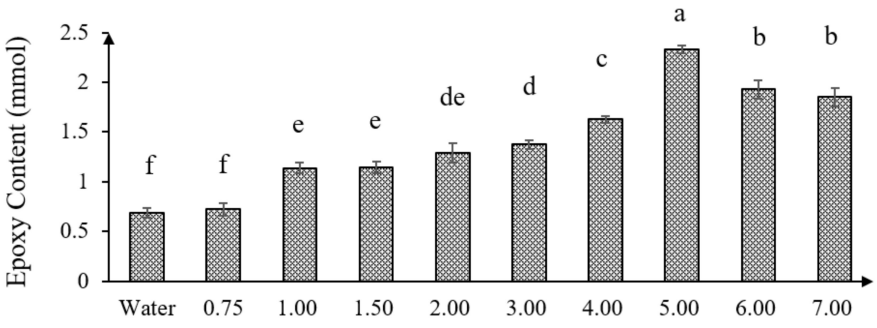


Figure 3. The reaction pathway of PVA epoxidation using epichlorohydrin.

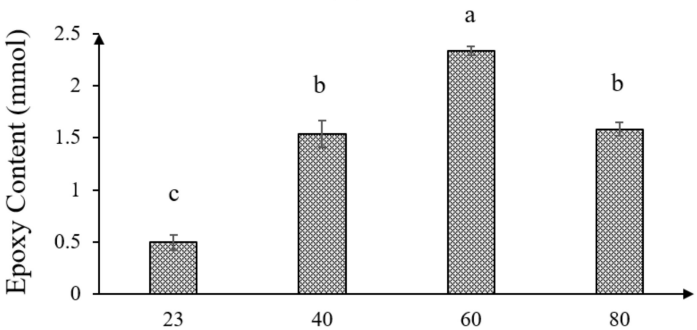
3.2.1. Impact of NaOH Concentration

During the PVA epoxidation process, NaOH plays a key role in dechlorination by forcing the replacement of chlorine with an epoxy group (Figure 3). In addition, NaOH functions to maintain an alkaline pH that is necessary for the substitution of hydroxyl groups. Based on conditions used for PEG diglycidyl ether synthesis [17], which has a similar reaction mechanism to PVA epoxidation, the impact of NaOH concentration on the epoxy content of the epoxidized PVA was studied (Figure 4A).

To study the impact of NaOH concentration on the PVA epoxidation, the reactions were carried out at different NaOH concentrations using a 1:3 (hydroxyl groups in PVA: epoxy groups in epichlorohydrin) molar ratio at 60 °C for 12 h. Compared with the water control, NaOH started to improve the overall epoxy content of the reaction product at a concentration of 1.00 mol/L (Figure 4A). In addition, the epoxy content of epoxidized PVA increased with increasing NaOH concentration up to 5.00 mol/L. Concentrations higher than 5.00 mol/L resulted in lower epoxy content in the product (Figure 4A), which may have been caused by self-polymerization of epoxidized PVA. Furthermore, it is worth noting that the colour of PVA solution was changed from colourless to yellow at high NaOH concentrations (7.00 mol/L) during the epoxidation. This was consistent with the colour change observed by Cecelia et al., which was reported to be caused by the hydrolysis of PVA [27]. Therefore, for subsequent reactions, NaOH was used at a molar concentration of 5.00 mol/L.



(**A**)



(**B**)

Figure 4. *Cont.*

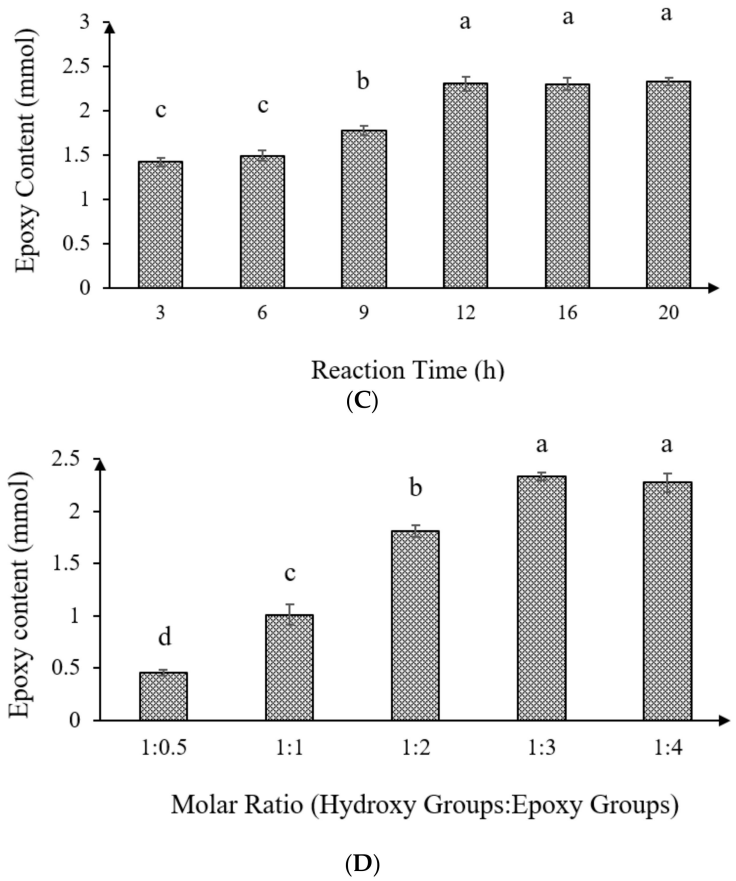


Figure 4. The impact of reaction parameters on the epoxy content of epoxidized PVA obtained from 1.14 g PVA epoxidation: (**A**) The impact of NaOH concentration; reactions were performed at 60 °C for 12 h, using a 1:3 (hydroxyl groups: epoxy groups) molar ratio. (**B**) The impact of reaction temperature; reactions were performed for 12 h, using a 1:3 (hydroxyl groups: epoxy groups) molar ratio and 5.00 mol/L NaOH. (**C**) The impact of reaction time; reactions were performed at 60 °C, using a 1:3 (hydroxyl groups: epoxy groups) molar ratio and 5.00 mol/L NaOH. (**D**) The impact of molar ratio (hydroxyl groups in PVA: epoxy groups in epichlorohydrin); reactions were performed at 60 °C for 12 h, using 5.00 mol/L NaOH. Milli-Q water (Water) was used as a negative control for this study. Values in the figure represent the average mean ± standard deviation of triplicate experiments. Values that do not share the same letter indicate that they are significantly different ($p < 0.05$).

3.2.2. Impact of Reaction Temperature

Temperature is another important parameter that affects the final equilibrium and reaction rate of the epoxidation process. To study the impact of temperature, the reactions were carried out at different temperatures at a 1:3 molar ratio (hydroxyl groups in PVA: epoxy groups in epichlorohydrin) for 12 h using 5.00 mol/L of NaOH. The results in Figure 4B indicated the increasing trend of epoxy content as the temperature increases from 23 °C to 60 °C. Furthermore, a decreased epoxy content was observed at 80 °C in Figure 4B. The newly generated epoxy groups in PVA molecule can further react with unreacted hydroxyl groups of PVA at base conditions, resulting in the self-polymerization of PVA and forming hydrogel as the final product [28–30]. It can be thus inferred that higher temperatures (≥80 °C) might intensify the self-polymerization of PVA, consuming epoxy groups and hydroxyl groups. Hence, 60 °C was the temperature used in subsequent PVA epoxidation.

3.2.3. Impact of Reaction Time

After examining the effect of epoxidation temperature and NaOH concentration, we then assessed the impact of reaction time. For these reactions, the reaction temperature was 60 °C, the NaOH

concentration was 5.00 mol/L, and the molar ratio was 1:3 (hydroxyl groups in PVA: epoxy groups in epichlorohydrin). As shown in Figure 4C, an increasing epoxy content in the epoxidized products was observed with increasing reaction time up to 12 h. At this point, the reaction reached equilibrium and no further increases in epoxy content were observed as the reaction time was increased to 20 h indicating the tendency of the epoxidized PVA to avoid self-polymerization. This can be explained by two possible reasons. On one hand, due to the consumed NaOH during epoxidation, the pH of the solution was decreased to 6.0 after 12 h, which is relatively lower than the pH 11–12 required for hydroxyl-epoxy coupling as reported by Greg et al. [15]. On the other hand, it is also possible that most of hydroxyl groups have already reacted and been substituted by the epoxy groups at 12 h, thus limiting self-polymerization. Taken together, a 12 h reaction time was employed for all future epoxidation reactions.

3.2.4. Impact of Molar Ratio

Finally, the effect of changing the molar ratio between the hydroxyl groups of PVA and epoxy groups of epichlorohydrin were studied. As shown by the results in Figure 4D, there was a strong correlation between the amount of epichlorohydrin used in the reaction and epoxy content in the reaction product. The maximum epoxy content was observed in the reaction where molar ratios of 1:3 and 1:4 were employed. However, there was no significant difference in epoxy content using these two ratios. Therefore, in subsequent reactions, a 1:3 molar ratio was used as this had a lower requirement for epichlorohydrin.

3.2.5. Summary of PVA Epoxidation

By using the most promising reaction conditions identified in this section (i.e., 5.00 mol/L NaOH, 60 °C for 12 h, and a molar ratio of 1:3), a stable poly-epoxide with an epoxy content of 2.33 ± 0.03 mmol was achieved in aqueous conditions from the epoxidation of 1.14 g PVA. This is the first report to describe a synthesis method for epoxidized PVA, offering an alternative strategy for PVA cross-linking with amines or other proteinaceous materials. Additionally, the characterized epoxy content makes reaction of epoxidized PVA with primary amine groups (0.59 ± 0.05 mmol/g) or carboxylic acid groups (1.69 ± 0.06 mmol/g) of SRM-derived peptides at a certain molar ratio of functional groups possible.

3.3. Synthesis of PEP Composites

In this section, epoxidized PVA was used as a cross-linker to potentially react with amines and carboxylic acid groups in SRM-derived peptides. To this end, several reactions were setup in which the amount of peptides used was varied to change the molar ratio of the reactions (Table 2).

3.3.1. Assessing the Degree of Cross-Linking through Primary Amine Group Quantification

Compared with other functional groups commonly found in peptides that can react with epoxides (i.e., –COOH, –OH, –SH; Figure 1), primary amino groups possess the highest nucleophilicity, and thus are most likely to react with epoxy groups [15]. Therefore, primary amino groups of SRM-derived peptides are likely the predominant target for the epoxide/PVA cross-linking reaction performed at alkaline pH (pH = 9.0, suggested by Shechter et al. [16]). Based on this assertion, the rate of cross-linking between SRM-derived peptides and epoxidized PVA was assessed through quantification of residual primary amine groups in the reaction products.

Role of Cross-Linking Temperature

Initial cross-linking studies were performed at various temperature to determine the most promising reaction temperature. As shown in Figure 5A, it was observed that relatively low levels of primary amino groups were observed in all the cross-linked products compared to the reaction with no epoxidized PVA, indicating the occurrence of the amine-epoxide cross-linking reaction. The lowest

levels of residual amines (0.03 ± 0.01 mmol/g) were observed in reactions performed at 23 °C (Figure 5A). However, as the reaction temperature was increased to 80 °C, there was a small increase in the amount of residual primary amino groups in the product, suggesting that higher temperatures have a slight inhibitory effect on the amine-epoxide reaction (Figure 5A). This may result as higher temperatures may favour competing reactions. For instance, a hydroxyl-epoxide reaction was reported to occur at 60–100 °C at a base condition in studies from Iulian et al. and Shechter et al. [20,31]. Therefore, 23 °C was used as the reaction temperature for subsequent cross-linking experiments.

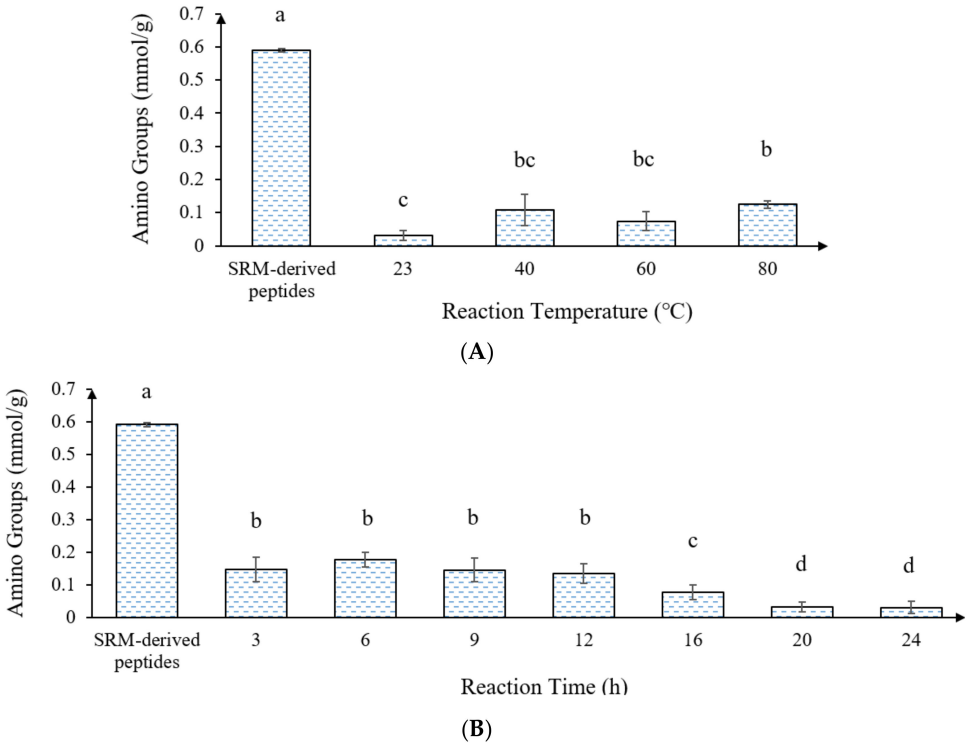


Figure 5. The role of reaction parameters on the primary amino groups remaining in the PEP cross-linked at a molar ratio of 1:1 (epoxy groups in epoxidized PVA: primary amino groups in SRM-derived peptides): (**A**) The role of cross-linking temperature; a reaction time of 20 h and a pH of 9.0 were employed for the reaction. (**B**) The role of cross-linking time; a temperature of 60 °C and a pH of 9.0 were employed for the reaction. A control reaction was performed in which SRM-derived peptides were added to 15.0 mL of Milli-Q water, in the absence of epoxidized PVA. Values represent the average mean ± standard deviation of triplicate experiments. Values that do not share the same letter are significantly different ($p < 0.05$).

Role of Cross-Linking Time

The next studies examined the role of cross-linking time on the reaction between SRM-derived peptides and epoxidized PVA. As shown in Figure 5B, an increase in reaction time generally correlated with lower levels of residual primary amino groups. This suggests a direct relationship between the reaction time and the degree of cross-linking. Increasing the reaction time from 20 to 24 h did not result in lower levels of residual primary amino groups, and thus subsequent cross-linking reactions were performed using a 20 h reaction time.

3.3.2. Assessing the Degree of Cross-Linking through Carboxylic Acid Group Quantification

The residual carboxylic groups in the products were also evaluated to assess the role of the carboxylic groups of the peptides in the cross-linking reaction. For this study, the reaction was performed at 23 °C for 20 h at a pH of either 5.0 or 9.0. An acidic pH was employed since it can promote the reaction of carboxylic acid groups with epoxides [16]. In contrast to previous experiments,

1.30 g of SRM-derived peptides were added to 20 mL epoxidized PVA to maintain a molar ratio of 1:1 (carboxylic groups in peptides: epoxy groups in epoxidized PVA). The control experiment was performed by adding 1.30 g of SRM-derived peptides to 20 mL water. Although carboxylic groups could theoretically react with epoxy groups and hydroxyl groups, the level of carboxylic groups in cross-linked products obtained at pH 5.0 and pH 9.0 (98.5% ± 1.0% and 98.2% ± 1.5%, respectively) remained unchanged compared to the control (97.4% ± 2.3% for pH 5.0 and 98.0% ± 1.4% for pH 9.0). This indicated a limited reactivity of carboxylic groups during the cross-linking. This is consistent with the 'low carboxylic acid-epoxide reactivity' observed in the study of Shechter et al. [16].

3.4. Application of PEP Products as A Tackifiers

The products obtained from amine–epoxide cross-linking were then subjected to a variety of testing to facilitate measurement of their tackifier qualities. For these studies, PEP products generated from different molar ratios (epoxy groups in epoxidized PVA: primary amine groups in peptides) were studied to determine whether this had an impact on their binding strength, thermal resistance, moisture-holding capacity and aqueous viscosity.

3.4.1. Compression Strength of Pucks Produced Using PEP

To assess the binding strength of the various PEP products, we employed a testing method previously described by our group [7]. Briefly, "pucks" are made using wood fibers (i.e., MulchMax 101) and an aqueous solution containing the tackifier of interest. A texture analyser was then used to determine the force required to break the resulting pucks, which is represented as the breaking force (N) as shown in Figure 6.

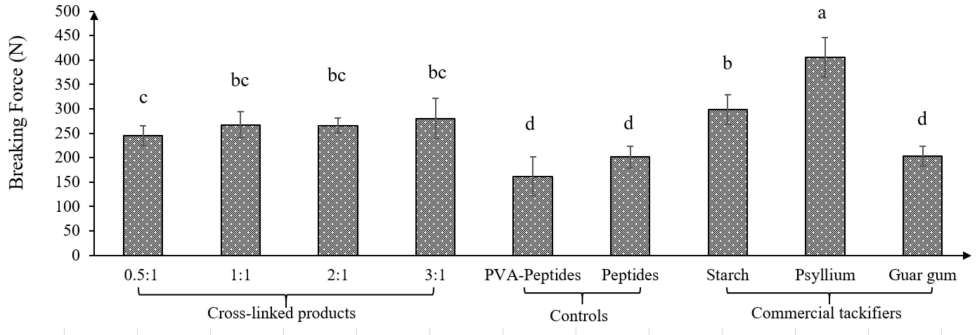


Figure 6. The compression strength of pucks. Pucks were prepared using MulchMax 100 wood fibres and a tackifier. The prepared pucks were cured at 40 °C for 48 h followed by a cooling process at room temperature for 24 h. The tackifier used was one of the cross-linked products (PEP generated at various molar ratios of epoxy groups: amino groups), controls (product obtained in reactions containing PVA-peptides product or SRM-derived peptides), or commercial tackifiers (starch, psyllium, guar gum). PVA-peptides (control) were obtained from the reaction between 1.14 g unmodified PVA and 4.00 SRM-derived peptides in 20 mL Milli-Q water at 23 °C and pH 9.0 for 20 h. The compression strength (i.e., breaking force (N)) of pucks were assessed using a *TA.XTPlus* texture analyser. The values shown represent the mean ± standard deviation of triplicate experiments. Values annotated with different letters are significantly different ($p < 0.05$).

The results in Figure 6 showed that all of the pucks generated using the PEP products displayed an enhancement in compression strength, when compared to those made with unmodified SRM-derived peptides or PVA-peptides products. Moreover, the compression strength of pucks made from cross-linked products generated at different molar ratios of epoxy groups: amino groups were statistically similar to each other, with all having higher compression strength than those generated using guar gum, a commercial tackifier. Furthermore, pucks generated at cross-linking molar ratios of 1:1, 2:1, and 3:1 displayed similar compression strength to pucks generated using starch. Taken together,

these data confirm the potential for the PEP product to replace guar gum and starch in commercial tackifier applications.

It should be noted that pure PVA and epoxidized PVA were also used as controls for these experiments. However, the pucks generated using these chemicals had no integrity and could not be subjected to compression strength testing. Thus, it is clear that these reagents are not capable of functioning as tackifiers themselves.

3.4.2. Thermal Resistance of PEP

TGA was then used to study the thermal resistance of PEP. This testing method can provide insight into the degree of cross-linking in the sample and can be used to infer whether changes to the molecular structure have occurred. During the TGA process, the masses of all samples remained constant in the early stages, followed by a dramatic decrease (decomposition stage) with increasing temperature (Figure 7). It can be observed that the PEP, obtained from the cross-linking at a 1:1 molar ratio (epoxy groups: amino groups), displayed a similar decomposition temperature as the SRM-derived peptides (around 250 °C), demonstrating that the PEP likely possessed a molecular structure that remained similar to the unmodified peptides (Figure 7). However, the PEP cross-linked at molar ratios of 2:1 and 3:1 (epoxy groups: amino groups) displayed a relatively constant and consistent decomposition stage, starting from 100 °C, which was right in between the thermal behaviour of epoxidized PVA and unmodified peptides (Figure 7). It can thus be inferred that the molecules of PEP cross-linked at molar ratios of 2:1 and 3:1 appeared to have more volatile components or structures imparted by the epoxidized PVA. Taken together, these data suggested the occurrence of cross-linking. It is interesting to note that, compared to the unmodified peptides, the products obtained from the cross-linking reaction at molar ratios of 2:1 and 3:1 had reduced thermal stability, which might limit the application of the PEP in other bio-industrial strategies.

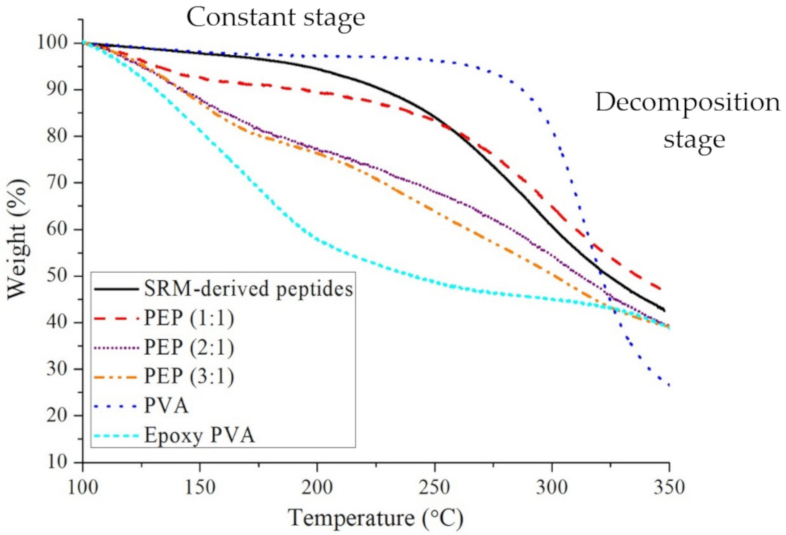


Figure 7. TGA. TGA was performed on SRM-derived peptides, PEP synthesized in cross-linking reaction at various molar ratios (1:1, 2:1, 3:1; epoxy groups: amino groups), PVA, and epoxidized PVA from 100 °C to 350 °C at a heating rate of 5 °C/ min. The powders of epoxidized PVA used in TGA were generated through lyophilization of the epoxidized PVA solution. Triplicates were applied for TGA and all results were reproduced to 5% error or better. Since the replicates for each material were similar, only one run is shown above.

3.4.3. Moisture-Holding Capacity of PEP

Moisture-holding capacity was also studied as this is an important property for tackifiers. This is defined as the ability to retain the water obtained from surface water, rains, and/or the hydro-mulching

process. The moisture retained provides seeds and plant with water to promote plant growth. As shown in Figure 8, epoxidized PVA powders demonstrated the highest moisture-holding capacity. This is likely due to the large number of hydroxyl groups present, which contributes to the relatively strong bonding with water. The PEP obtained from the cross-linking reaction at 2:1 and 3:1 molar ratios (epoxy groups: amino groups) displayed increased water-holding ability compared to the unmodified peptides and the product obtained from the cross-linking reaction performed at a 1:1 molar ratio. This can be explained by the relatively high proportion of epoxidized PVA molecules that have been integrated into the product cross-linked at 2:1 and 3:1 ratios. Furthermore, the values measured for the products obtained from cross-linking reactions at 2:1 and 3:1 ratios were higher than that of starch and psyllium, and similar to the levels achieved by guar gum. This shows the competitiveness of PEP with commercial tackifiers with regards to water retention capacity. It is worth noting that even though PEP cross-linked at a 1:1 ratio exhibited a reduced moisture retention (Figure 8), the values achieved were statistically similar to those of starch and psyllium. The different water-holding capacities achieved for the various cross-linked products (PEP) also highlight the versatility of using peptide-derived tackifiers, enabling manufacturers a mechanism to modulate cross-linking ratios to produce tackifiers suited for a variety of geographical areas with different moisture retaining requirements. For instance, in areas where a significant amount of rain is common, PEP cross-linked at a 1:1 ratio may be more applicable as water-holding capacity would not be as important. On the other hand, the outstanding water-holding capability of PEP products cross-linked at 2:1 and 3:1 ratios can potentially help retain the necessary moisture for germination and growth of vegetation in drier geographic locations (i.e., the desert).

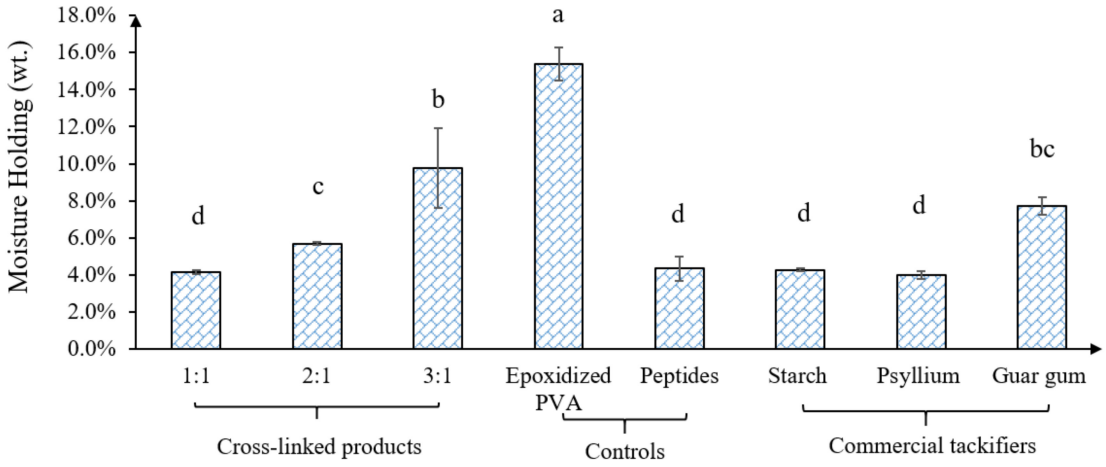


Figure 8. The water-holding capacity of peptides, PVA, PEP cross-linked products, and commercial tackifiers. 5% (w/v) solutions of the sample powders were added to Petri dishes, which were then placed in an incubator at 22.0 °C and 0.0% relative humidity for 60 h. The moisture remaining after 60 h was determined and used to calculate the moisture-holding capacity of the residual material. For the cross-linked products, the molar ratio (epoxy groups in epoxidized PVA: primary amino groups in peptides) employed during the cross-linking reaction is indicated (i.e., 1:1, 2:1, or 3:1). Epoxidized PVA powders used for the test were generated via lyophilization of the epoxidized PVA solution. Peptides represent the unmodified SRM-derived peptides. The values shown represent the mean ± standard deviation of triplicate experiments. Values annotated with a different letter are significantly different ($p < 0.05$).

3.4.4. Aqueous Viscosity of PEP

Aqueous viscosity of the tackifiers is another essential parameter, which affects the strategy for tackifier application, the rate of fibre mixing, and the spraying distance during the hydro-mulching process. Tackifiers with low aqueous viscosity are generally preferred, as this reduces fluid drag force during mixing and spraying and also minimizes the clogging of the hydro-mulching equipment. As indicated by the results of viscosity measurement, there was no significant difference between the

viscosity of the aqueous suspensions of any of the cross-linked products, which were 1.00 ± 0.17 cP, 0.97 ± 0.15 cP, and 1.00 ± 0.10 cP for the material generated from the cross-linking at 1:1, 2:1, and 3:1 ratios (epoxy groups: amino groups), respectively. Moreover, when compared with psyllium (1.50 ± 0.26 cP) and guar gum (~ 26.0 ± 2.43 cP), the SRM peptides and the various PEP products displayed a lower aqueous viscosity in suspension, which provides further evidence that the peptides-based products are promising commercial tackifiers for hydro-mulching.

3.4.5. Estimated Cost of Tackifiers Derived from Peptides

A complete techno-economic assessment for production of PEP cannot currently be completed, as the process has not been optimized nor scaled-up to pilot scale. However, the SRM used to generate PEP is of negative value, which greatly strengthens the process economics. Rendering companies pay ~US$0.2/kg for SRM disposal, and thus it may be possible for manufactures to collect tipping fees to dispose of the primary feedstock for PEP [7]. According to Alibaba, epichlorohydrin (99.0%) and PVA (industrial grade) can be purchased for only US$1.0/kg and US$1.1/kg, respectively [32,33]. Meanwhile, the hexane used during the process will likely be recycled via evaporation. Taken together, the low cost of the reactants suggests that EPC-PVA-Peptides will likely be competitive with the price for psyllium (US$1.67/kg), starch (~US$1.00/kg), and guar gum (US$18.00/kg) [11,14].

4. Conclusions

By facilitating the cross-linking between SRM-derived peptides and epoxidized PVA, a peptides-based tackifier was successfully developed and characterized. As a peptides-based product with great potential, all the PEP products displayed comparable binding strength with commercial tackifiers, as well as a relatively low aqueous viscosity and feedstock price. In addition, by adjusting the molar ratio of the cross-linking reagents, the water-holding capacity for PEP can be varied to cater to different applications. Through this study, a novel pathway is demonstrated for the bioconversion of SRM-derived peptides into novel tackifiers, which may be applied to other cross-linking studies involving PVA and amines.

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Conflicts of Interest: The authors declare no conflict of interest.

Data Availability: The raw/processed data required to reproduce these findings are available to download from http://dx.doi.org/10.17632/nwcbpmkbzy.1.

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