

Effects of fiber blending and diamines on wheat gluten materials reinforced with hemp fiber

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Received: 21 November 2009 / Accepted: 12 April 2010 / Published online: 4 May 2010
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Abstract Wheat gluten (WG) is a promising base material for production of “green” plastics, although reinforcement is needed in more demanding applications. Hemp fiber is a promising reinforcement source but difficulties exist in obtaining desired properties with a WG-based matrix. This study aimed at improving fiber dispersion and fiber–matrix interactions using a high speed blender and a diamine as a cross-linker. Samples were manufactured using compression molding, two types of blenders and addition of diamine. Mechanical properties were assessed with tensile testing. Tensile-fractured surfaces were examined with scanning electron microscopy (SEM). Protein polymerization and fiber–protein matrix interactions were examined using high performance liquid chromatography (HPLC) and confocal laser scanning microscopy (CLSM). The results showed that a higher-speed grinding yielded a more even distribution of fibers and a more polymerized protein structure compared to a lower-speed grinding. However, these improvements did not result in increased strength, stiffness, and extensibility for the higher-speed grinding. The strength was increased when the grinding was combined with addition of a diamine (Jeffamine[®] EDR-176). HPLC, SEM, and CLSM, indicated that diamine added samples showed a more “plastic” appearance together with

a stiffer and stronger structure with less cracking compared to samples without diamine. The use of the diamine also led to an increased polymerization of the proteins, although no effect on the fiber–protein matrix interactions was observed using microscopical techniques. Thus, for future successful use of hemp fibers to reinforce gluten materials, an appropriate method to increase the fiber–protein matrix interaction is needed.

Introduction

Increasing environmental waste problems, such as the plastic “continent” floating around in the Pacific Ocean (<http://www.greenpeace.org/international/footer/search?q=plastic+trash+in+the+ocean>), have led to increasing demand for environmentally friendly plastic materials to be used by the industry. Also, the depletion of petroleum resources calls for development of high performance bio-based plastics and composite materials from non-petroleum sources such as, e.g., plants [1]. One of the most interesting alternatives for traditional synthetic plastics is wheat gluten (WG)-based bioplastics, due to the combination of mechanical, oxygen barrier (drier environments), and film-forming properties in those types of bioplastics [2–4]. Reinforcement of the bioplastics by biofibers may lead to a wider range of applications for the material. Plants with interesting fibers that have been used to reinforce bioplastics include flax, hemp, jute, coconut, and nettles [5]. High strength and elastic modulus, low density, non-abrasiveness, and biodegradability are the main advantages of using plant fibers [6].

A number of studies have been carried out in order to investigate possibilities to reinforce plastics by the use of plant fibers [5]. However, until now, only few studies have

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focused on the use of plant fibers for reinforcement of bioplastics, and most of them concern plant fibers in soy-based materials [7, 8]. Gluten material composites have been evaluated by using, e.g., silica [9], hydroxyethyl cellulose [10], and methylcellulose [11] as microfibrils. Recently, possibilities to reinforce plasticized WG materials by hemp fibers have been investigated in two independent studies [12–14]. The first study evaluated mechanical improvement and deplasticizing effect of the use of natural fibers to reinforce gluten materials [12], as well as influence of thermal treatment on fiber/matrix adhesion [13]. The second study evaluated improvements in mechanical properties of enforcing wheat materials using long and short hemp fibers [14]. Generally, the main problems with using plant fibers to reinforce bio-based plastics are that the fibers tend to cluster together during mixing, thereby causing weaker unreinforced zones in the material. Another problem reported by several authors is a poor bonding between the natural fiber and the matrix [7, 14, 15]. To address the problem of poor bonding, different strategies have been proposed, such as addition of alkali solutions to decrease the inter-fibrillar region by removing hemicellulose and lignin [15] or addition of compatibilizers such as polyester amide grafted glycidyl methacrylate [7]. Both types of additives have been shown to lead to a better and more homogeneous dispersion of fibers in the material and to improvement in composite impact strength [7, 15]. No investigations have evaluated possibilities for better dispersion and bonding of plant fibers in WG-based bio-based plastics in order to improve their mechanical properties.

The aim of the present study was to investigate possibilities of improving hemp fiber distribution in wheat gluten-based matrices (WGM), as well as the bonding between the fibers and the WGM. The hypothesis was that (1) increased mixing speed and (2) addition of a cross-linking agent would lead to improved distribution of fibers in the matrix, a better incorporation of fibers in the matrix and improved mechanical properties. Thus, two different mixing procedures were evaluated as well as the addition of a diamine.

Materials and methods

Materials

Powder of WG, consisting of 84.8 wt% WG proteins, 8.1 wt% wheat starch, 5 wt% water, 1.34 wt% fat, and 0.76 wt% ash, was supplied by Reppe AB, Lidköping, Sweden. Karlshamn's Tefac AB, Karlshamn, Sweden, supplied glycerol (glycerol > 99.5 wt%, water < 0.5 wt%). The diamine, Jeffamine[®] EDR-176 (polyetheramine), was

provided by Huntsman Holland BV, Holland. Jeffamine[®] polyetheramine has the following structure:



Industrial hemp was cultivated in Southern Sweden and fibers of three different quality types were selected as in Wretfors et al. [14].

- Hemp fiber—poor quality: Unretted hemp stalks, harvested by forage harvester, were partially dried in plastic tubs with fans placed underneath the hemp material. The resulting fiber was heavily retted and designated ‘poor’ quality.
- Hemp fiber—standard quality: Unretted hemp stalks, harvested by forage harvester, were processed through a hammer mill with sieve size 50 mm (Kamas Industri AB, Sweden) and then through a step cleaner (Hergeth GmbH, Germany) to remove larger size shives. The resulting fiber type was designated ‘standard’ quality.
- Hemp fiber—good quality: Unretted hemp stalks, harvested by forage harvester, were processed through a hammer mill (Kamas Industri AB) without any sieve installed and then through a step cleaner (Hergeth GmbH, Germany) to remove large and small shives. Because no sieve was used, the fiber passed quickly through the mill, resulting in less fiber damage. The resulting fiber type was designated ‘good’ quality.

Size distribution of fibers

In order to evaluate size distribution of the fibers of different qualities, light microscopy images were produced from the same samples as in Wretfors et al. [14]. From each sample (poor, standard, and good quality) three different representative areas were selected on the images and width of fibers was measured according to Wang et al. [16]. The software Leica QWin/V3 was used for the measurements. One hundred fibers of each representative area were measured. The size distribution of fiber width of each sample was thereafter evaluated.

Sample preparation for compression molding

Gluten and glycerol (30 wt% based on the weight of gluten and glycerol) were mixed into a dough that was mortared for 5 min at about 150 rpm using a Mortar Agate from VWR International. Any woody substance was removed manually from the hemp fibers, and thereafter the fibers were processed in a high speed grinder (model A10, IKA-WERKE, Staufen, Germany) for 2×10 s. The resulting

fibers were very short in order to facilitate an even distribution in the plastic material [14]. Samples of hemp fiber-reinforced WGM were made through additions of 5, 10, 15, and 20 wt% of poor, standard, and good quality hemp fibers to the gluten dough. Dough and fibers were transferred either to an electrical blender (Waring Commercial, USA), or to the high speed grinder (model A10, IKA-WERKE, Staufen, Germany). The gluten dough together with the fibers were mixed in the electrical blender and in the high speed grinder, using several short bursts, until the fibers and gluten dough were considered sufficiently mixed for compression molding. For the high speed grinder, several separate portions had to be mixed (number of portions depended on amount of fiber added) in the grinder and then combined to make compression molded specimen.

A diamine (Jeffamine[®] EDR-176 polyetheramine, 1.0 wt% relative to the weight of the dough) was added to some fiber-dough samples prior to mixing. In addition, a sample containing 2.0 wt% diamine was also manufactured.

Compression molding

By the use of a PHI press (Pasadena Hydraulics Inc, California, USA), compression molded films were processed [14]. A frame was used resulting in square films (100 × 100 mm², thickness 0.5 mm) and Mylar[™] foils were used between the plates and the sample. The thickness of the frame (resulting in the thickness of the films) was selected similar as has been used in previous investigations of pure gluten films [17] in order to be able to compare the results of tensile measurements. As fibers are known to have a fiber thickness of 106–139 μm [18], the sample thickness of 0.5 mm was expected to be enough of being comparable in terms of tensile properties with previous samples. Molding temperature was 130 °C at set pressure of 100 bar led to a pressure of 1600 bar with molding time of 5 min. The plates were removed from the press after molding and then the films were left to cool to ambient temperature. Finally, the Mylar[™] foils were removed and the films were separated from the frame using a scalpel. Sample thickness was measured according to Wretfors et al. [14].

Tensile testing

Mechanical properties of the samples were measured using a Zwick Z010 tensile strength tester (ZwickRoell) equipped with a 500 N load cell controlled by a testXpert 7.1 (Lambda Instruments AB) according to ISO 527-3:1995(E) [14]. An initial grip distance of 40 mm was used together with a crosshead speed of 100 mm/min. The strain was estimated from the relative clamp movements using 40 mm as the initial length. The stress was measured as the force

divided by the narrow initial cross-section. The Young's modulus was estimated from the initial slope of the stress-strain curve. A pre-load of 0.2 N was used. Of each sample without diamine, a total number of 15 specimens were tested, while for samples containing diamine, 13 replicates were taken due to a smaller supply of materials available.

Scanning electron microscopy (SEM)

Tensile fractured surfaces of hemp fiber-reinforced WGM were studied using an LEO 435VP scanning electron microscope (Cambridge, UK) with a secondary electron detector at acceleration voltage of 10 kV. Samples were mounted on the stubs and sputtered with an Au/Pd 3:2 coating (JFC-1100, JEOL, Tokyo, Japan). Plastics samples from each of the treatments were selected and the actual fracture surfaces of the samples were observed in order to study the fiber fracture and distribution.

Confocal laser scanning microscopy (CLSM)

Immunostaining

Confocal laser scanning microscope with immunostaining of the proteins was carried out, building on methods developed at INRA-Nantes (unpublished results) with modification. Small pieces of gluten bioplastics film sections, size of approximately 2 × 2 mm (thickness of approximate 0.5 mm), were blocked in 10% (w/v) bovine serum albumin (BSA) in TBS-T (20 mM Tris, 500 mM NaCl, 0.3% Tween[®] 20; pH 7.5) buffer at room temperature for about 16 h. After blocking, the pieces of gluten bioplastics were incubated with a mixture of two primary antibodies, (1) high molecular weight glutenin subunits (HMW-gs) antibody (monoclonal, mouse, 1601, a synthetic peptide GSVTCPQQV, diluted 1:1, courtesy to C. Mills, Institute of Food Research, UK) specific for subunits 2, 5, 10, 12 and (2) gliadin (Glia) antibody (polyclonal, antiserum (IgG), rabbit, diluted 1:500, Sigma, St. Louis, MO, USA) in 10% (w/v) BSA blocking and Tris-buffered-saline (pH 7.5) of equal volumes of solutions. Specimens were incubated with both primary antibodies on the rotator for about 72 h at room temperature. After washing 4 × 30 min in TBS-T, the samples were incubated 21 h at room temperature with a mixture of two secondary goat antibodies, an anti-mouse antiserum (IgG) conjugated to Alexa 488 and anti-rabbit antiserum (IgG) Alexa 546, both diluted (1:50) in dilution buffer; then washed 3 × 30 min in TBS-T and 3 × 15 min in distilled water, and replaced on the object slides in a drop of water with a cover slip to be viewed by fluorescence and confocal laser scanning microscope.

Image processing

Immuno-stained specimens were imaged with a laser scanning confocal microscope (Zeiss LSM META 510, Germany) equipped with a 40× oil-immersion objective. In gluten bioplastics the HMW-gs (Alexa 488) and gliadin (Alexa 546) labeled structures were excited using an Argon laser at 488 nm and HeNe lasers at 543 nm. Emitted fluorescence was detected using 505 and 560 nm long-pass filters, respectively. Single optical images were scanned at resolution 1024 × 1024 pixels and converted into high resolution TIFF images.

HPLC

Protein solubility in the films was determined using size-exclusion high performance liquid chromatography (SE-HPLC) according to the three-step extraction procedure developed by Gällstedt et al. [17]. In this method, proteins are extracted from a sample in three subsequent steps. In the first step, the sample is treated with an extraction buffer of dilute sodium dodecyl sulfate (SDS), and thus proteins soluble in this buffer are extracted. The extraction buffer together with the proteins is collected and a new extraction buffer of SDS is added to the sample which is thereafter treated with a short sonication in an ultrasonic disintegrator (Soniprep 150, Tamro, Mölndal, Sweden) for 30 s, amplitude 5, fitted with a 3 mm exponential microtip. The extraction buffer together with the proteins is again collected and a new SDS extraction buffer is added to the sample which is treated with repeated sonication (30 + 60 + 60 s sonication) before the sample buffer together with extracted proteins is again collected. Thus, from each of the samples, three different protein extractions are obtained. The proteins from each sample and extraction were thereafter separated by SE-HPLC analyses using a Waters HPLC system using a BIOSEP SEC-4000 Phenomenex column as described in Johansson et al. [19]. The areas under the chromatograms were thereafter used as measurements of amounts of proteins from the different extractions [19]. The total amount of protein extracted from the pure gluten/glycerol plastic samples was used as a standard sample and was normalized to the value 100 [20]. Protein in pure gluten powder has been shown to be fully extractable after the three extractions described above [17], although as soon as the proteins are crosslinked by treatments used to produce materials, 100% of the proteins is very difficult to extract [20]. The amount of proteins extracted after each extraction step in the other samples was then normalized to the total protein solubility of the pure WG/glycerol plastics. Similarly as has been found in previous investigations, protein extractability in some materials showed higher values as

compared to the pure WG/glycerol plastics, probably due to changes in the conformation of the proteins [20].

Statistical analysis

For the statistical analyses, SAS software package for Microsoft Windows (SAS Institute Inc, NC, USA) was used. Regression analyses, general linear model (GLM), and analyses of variance (ANOVA) were carried out, followed by calculation of means for the different treatments with determination of significance using LSD (0.05).

Results

Fiber size distribution

Size distribution, measured as width, of the fibers in the used fiber quality classes was found to vary substantially, from 0.01 to 1.17 mm. However, most fibers had a width of below 0.15 mm and no significant differences in size distribution was found among the fiber quality samples recorded (Fig. 1).

Fiber distribution

SEM indicated that the use of a high speed grinder improved the fiber distribution (Fig. 2a) in comparison with the use of an electrical blender (Fig. 2b). The fractured surface was cleaner and more even in samples blended with high speed grinder than with electrical blender, although a somewhat more spread of the fibers along the fracture surface was observed. The images shown represent standard quality fibers as no obvious difference in fiber distribution was seen between different quality fibers. However, the use of the high speed grinder led to inferior

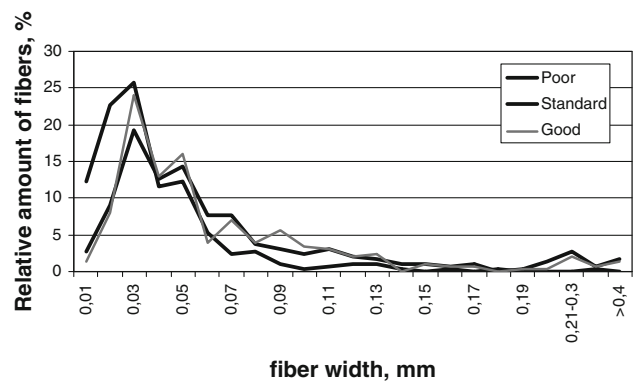


Fig. 1 Size distribution of fibers in samples from the different fiber quality samples (poor, standard, and good). The variation in width is large in all three fiber quality classes and no significant difference was observed in size distribution among the quality classes

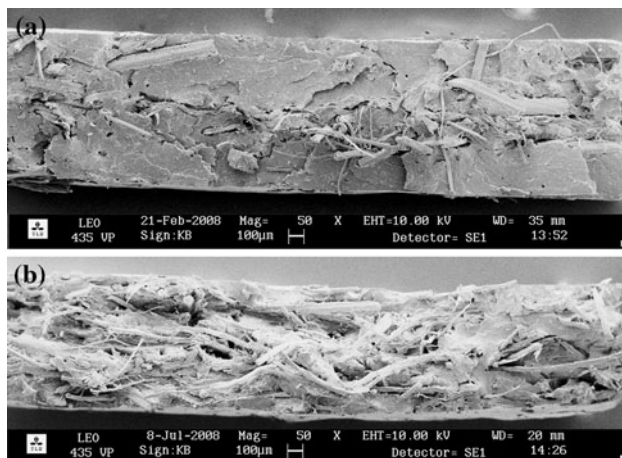


Fig. 2 SEM images showing the fractured surfaces of WGm reinforced with short hemp fibers of standard quality: **a** dough and fibers mixed with the high speed blender, **b** dough and fibers mixed with the electrical blender. The scale bar corresponds to 100 μm

mechanical properties (lower strength, stiffness, and extensibility) compared with using the more “gentle” electrical blender (Table 1). The scatter (standard deviation) in the mechanical data was large (average 38 and 30% for, respectively, Young’s modulus and fracture strain), for all sample types, even for those with improved fiber distribution (Table 2).

Addition of diamine

SEM analyses gave no indications of a better bonding between the hemp fibers and the protein WGm when the diamine was added (compare Fig. 3b with Fig. 3a). In both cases, the existence of several fibers been pulled out during the tensile testing indicated insufficient bonding between the fiber and the matrix (Fig. 3). Also, CLSM did not indicate any differences in bonding between the hemp fibers and the protein matrix for samples with or without addition of the diamine (Fig. 4d compare Fig. 4c). For both type of samples, the fibers were stained red (red fluorescence is likely due to the secondary antibody precipitation) with green stain along the fibers indicating presence of HMW glutenin subunits. Thus, for both types of samples, a

Table 2 Mean Young’s modulus, fracture strain, and fracture stress of hemp fiber reinforced gluten plastics mixed with the high speed grinder and with and without Jeffamine additions, containing different fiber amounts and qualities

Treatment	Young’s modulus (MPa)	Fracture strain (%)	Fracture stress (MPa)
WGm	32.1 (4)	126.8 (9)	3.7 (0.3)
5% fiber, poor	33.4 (12)	34.2 (11)	1.5 (0.4)
10% fiber, poor	67.8 (30)	15.5 (4)	2.8 (1.1)
15% fiber, poor	79.2 (28)	11.2 (3)	2.5 (0.8)
20% fiber, poor	78.0 (22)	10.6 (2)	2.7 (0.9)
5% fiber, average	74.2 (46)	20.3 (5)	2.3 (1.1)
10% fiber, average	81.6 (29)	15.4 (6)	2.7 (0.9)
15% fiber, average	88.1 (40)	13.5 (6)	3.0 (1.1)
20% fiber, average	92.7 (24)	9.2 (2)	3.4 (1.0)
5% fiber, good	42.9 (17)	32.3 (12)	1.7 (0.5)
10% fiber, good	55.7 (21)	15.6 (4)	1.9 (0.6)
15% fiber, good	88.0 (26)	11.5 (4)	2.6 (0.6)
20% fiber, good	104.6 (38)	9.0 (3)	3.2 (0.9)
WGm, Jeffamine	46.5 (3)	115.6 (5)	4.0 (0.1)
WGm, 2 \times Jeffamine	49.4 (5)	114.2 (6)	3.7 (0.2)
5% fiber, Jeffamine	103.5 (34)	19.0 (6)	3.1 (0.6)
10% fiber, Jeffamine	84.7 (21)	12.0 (3)	3.0 (0.6)
15% fiber, Jeffamine	135.0 (28)	8.7 (3)	3.9 (0.7)
20% fiber, Jeffamine	149.6 (33)	8.0 (2)	4.8 (0.7)
10% fiber, 2 \times Jeffamine	71.0 (11)	11.4 (3)	2.6 (0.6)

Standard deviation is shown within parenthesis

2 \times Jeffamine refers to 2 wt% Jeffamine as compared to 1 wt% in the other samples with Jeffamine

gathering of HMW glutenin subunits were found along the border of the fibers. To conclude no obvious differences were detected between the two samples as related to bonding between the hemp fibers and the protein WGm.

The tensile testing showed that the addition of the diamine to the material increased the strength and stiffness of the material (Young’s modulus, maximum stress, and fracture stress; Table 3; Fig. 5). Although the SEM analyses did not show an increased bonding between the fiber and the protein matrix, a noticeable change in the structure of the matrix itself, with less cracking and a more “plastic”

Table 1 Mean values of tensile strength measurements depending on mixing procedure

Source	Maximum stress (MPa)	Strain at maximum stress (%)	Fracture stress (MPa)	Fracture strain (%)	Young’s modulus (MPa)
Mix 1	3.6a	16.7a	2.7a	21.3a	112.1a
Mix 2	3.2b	12.5b	2.5a	16.5b	73.9b

Samples of all fiber quality and content were included in the statistical analyses and compared pairwise. No samples with added Jeffamine were included in the statistical analyses

Means with the same letters within a column do not differ significantly (LSD 0.05)

Mix 1 mixing with the electrical blender, Mix 2 mixing with the high speed grinder

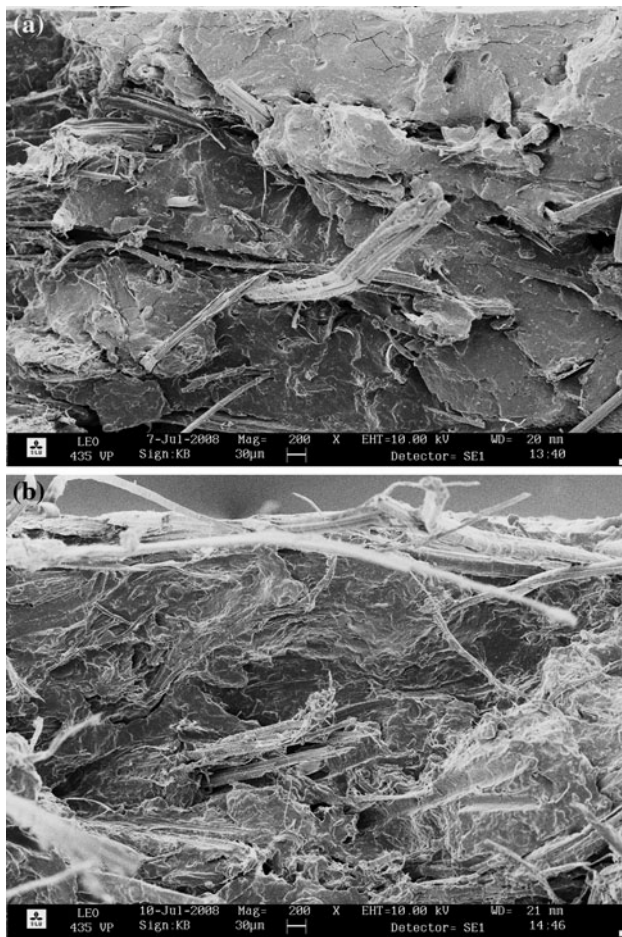


Fig. 3 SEM images of WGm reinforced with short hemp fibers of standard quality: **a** without diamine, **b** with diamine. The scale bar corresponds to 30 μm

with smooth continuous matrix surface was obvious (Fig. 3). Still, however, the standard deviations for the measured tensile parameters were large (mean 23 and 26% for, respectively, Young's modulus and fracture strain, Table 2).

SE-HPLC analyses showed a reduction in the degree of extraction of proteins when the diamine was added. This extractability reduction could be seen both as a decrease in the total extraction of proteins but also in the fact that of those proteins extracted, a relatively higher degree were the ones that were most easily extracted (i.e., extraction 1 related to extraction 3; Fig. 6). The reduction was most dominant in hemp containing samples for extractions 2 and 3, in which sonication was used to extract the proteins. The low extraction with sonication, which breaks disulfide bonds, indicates a more polymerized protein structure. Thus, the diamine seemed to increase the protein polymerization in the material. Addition of hemp fibers to the gluten led to an increase in protein extractions compared to pure WGm, indicating a weaker and less polymerized

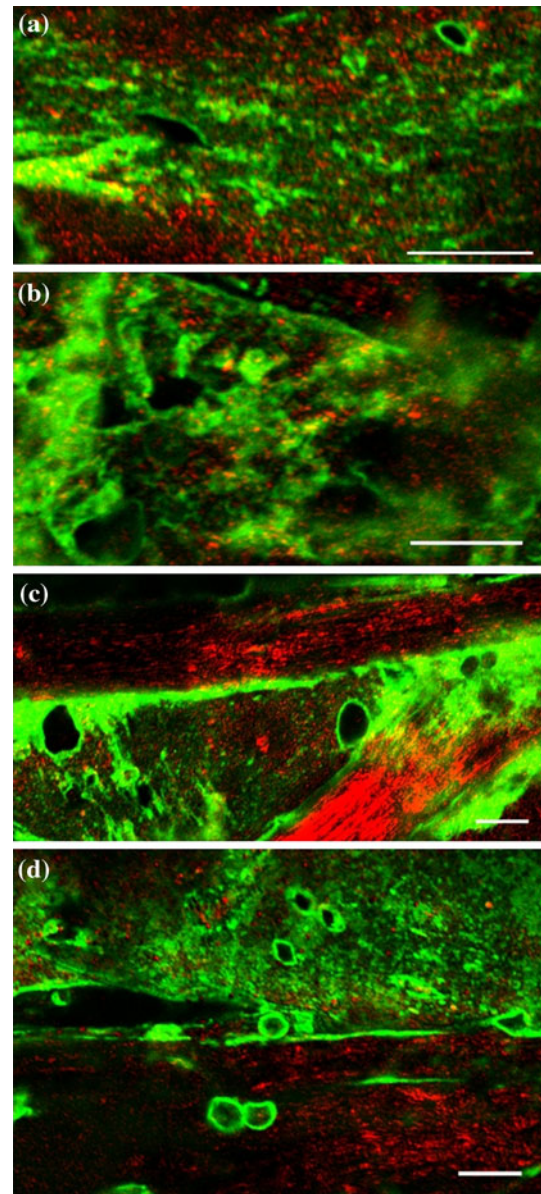


Fig. 4 CLSM images of WGm reinforced with short hemp fibers of standard quality: **a, c** without diamine; **b, d** with diamine; **a, b** showing only WGm; **c, d** showing WGm with fibers. HMW glutenin subunits (2, 5, 10, and 12) are labeled with green, whereas gliadins and fibers are labeled with red fluorescent dyes. The scale bar corresponds to 10 μm

structure (Fig. 6). Total protein extractability in some materials showed higher values as compared to those of the pure WG/glycerol plastics, probably due to changes in the conformation of the proteins [19].

Similarly to the results from the SE-HPLC analyses, also the results from the CLSM analyses indicated a more polymerized and bonded structure of the protein matrix in the diamine samples as compared to samples without diamine. A more polymerized and bonded gluten structure is characterized by a well established glutenin network and

Table 3 Mean values of tensile parameters with and without diamine in hemp fiber samples

Source	Maximum stress (MPa)	Strain at maximum stress (%)	Fracture stress (MPa)	Fracture strain (%)	Young's modulus (MPa)
No Jeffamine	3.55b	11.1a	2.82b	14.6a	86.8b
Jeffamine	4.49a	8.7b	3.68a	11.9b	118.2a

Mixing was carried out using the high speed grinder

Corresponding samples with 5–20% fibers, with and without Jeffamine are the ones included and compared with each other

Means with the same letters within a column do not differ significantly (LSD 0.05)

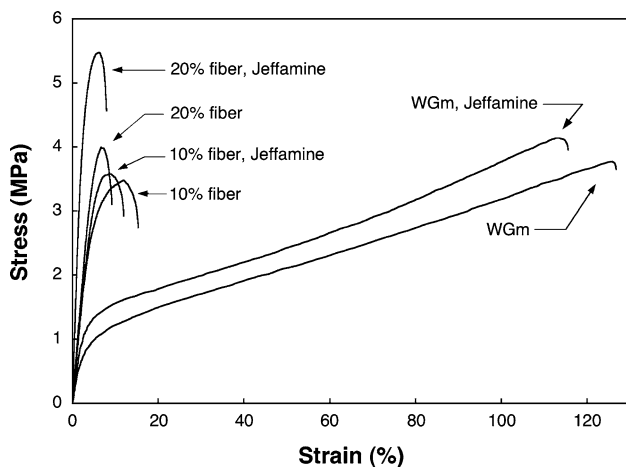


Fig. 5 Stress–strain curves of the gluten plastics. All the fibers correspond to the same average quality

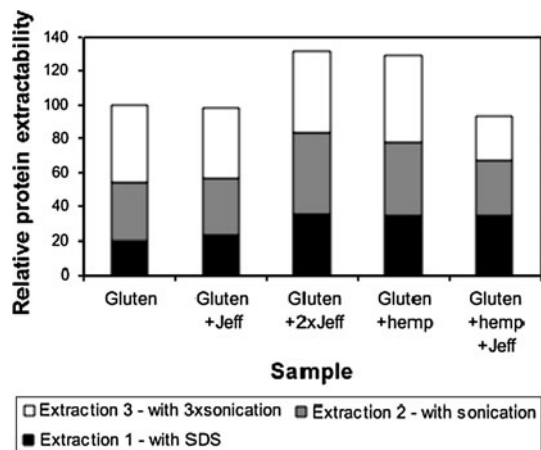


Fig. 6 Relative protein extractability of proteins from different protein extractions from the different samples. Protein amounts are determined by SE-HPLC and normalized towards extraction values of the pure gluten/glycerol samples. *Gluten* pure gluten/glycerol samples, *Gluten + hemp* Gluten reinforced by hemp fibers, *Gluten + hemp + Jeff* Gluten reinforced by hemp fibers and Jeffamine. Corresponding samples with 5–20% fibers, with and without Jeffamine are the ones included and compared with each other and with the pure gluten samples. 2× Jeff refers to 2 wt% Jeffamine as compared to 1 wt% in the other samples with Jeffamine

evenly spread and trapped gliadins. With the CLSM method used in this investigation, the HMW glutenin subunits are stained green and the gliadins are stained red. Clear differences were seen between the samples with diamine (Fig. 4b, d), in which the HMW glutenins (green fluorescence) were observed more dominating and with even fluorescence, while the gliadins (red fluorescence) were less evenly distributed within the structure, as compared to in the samples without diamine (Fig. 4a, c). Thus, the CLSM images of WGm structures with diamine provided an indication of the presence of a more bonded structure in the protein matrix as compared to those without diamine (Fig. 4).

Influence of different parameters on tensile strength

Regression analyses were carried out in order to compare importance of the different parameters used (i.e., grinding method, hemp fiber content and quality, and addition of diamine) for the various tensile parameters (i.e., maximum stress, strain at maximum stress, fracture stress, fracture strain, and Young's modulus). Fiber quality was found to play absolutely the least role for all of the tensile strength parameters (0.1–0.8% of the variation in any of the tensile strength parameters were explained by the fiber quality). Also the grinding method played relatively little role in determination of any of the tensile strength parameters (2.4–3.2%), except for the Young's modulus of which the grinding explained 7.0% of the variation. The impact of the diamine was highest for the maximum stress (explaining 12.7% of the variation), fracture stress (14.1%), and Young's modulus (14.2%) when added to samples containing hemp fibers. Addition of diamine to pure gluten samples increased the Young's modulus with 45% (Table 2). The most important factor of those investigated in explaining the variation in tensile parameters was hemp fiber content. The hemp fiber content explained 23.4% of the variation in maximum stress, 40.6% of the variation in strain at maximum stress, 19.0%/fracture stress, 46.2%/fracture strain, and 24.8% of the variation in the Young's modulus. The highest values in maximum stress, fracture stress, and Young's modulus occurred with a combination

Table 4 Mean values of tensile parameters as a function of content of hemp fiber and with/without diamine

Source	Maximum stress (MPa)	Strain at maximum stress (%)	Fracture stress (MPa)	Fracture strain (%)	Young's modulus (MPa)
Jeff, 5hemp	4.1c	12.0b	3.1d	19.0b	103.5b
Jeff, 10 hemp	3.6d	9.7c	3.0d	11.8e	84.7cd
Jeff, 15hemp	4.8b	6.7d	3.9b	8.7g	135.0a
Jeff, 20 hemp	5.5a	6.5d	4.8a	8.0h	149.6a
No, 5hemp	3.0e	14.7a	2.3f	20.3a	74.2d
No, 10hemp	3.5d	11.5bc	2.7e	15.4c	81.6cd
No, 15hemp	3.7cd	10.5bc	3.0d	13.5d	88.1c
No, 20hemp	4.0c	7.7d	3.4c	9.2f	92.7bc

Mixing was carried out using the high speed grinder

Jeff addition of diamine; *No* no addition of diamine; *5hemp*, *10hemp*, *15hemp*, *20hemp* % additions of hemp fiber of standard quality

Means with the same letters within a column do not differ significantly (LSD 0.05)

of the diamine and a high content of hemp fiber (Table 4). In contrast, high extensibility (strain at maximum stress and fracture strain) was obtained without diamine and using low fiber contents. In fact, the highest values were obtained without fiber [14].

Discussion

The use of the high speed grinder improved the hemp fiber distribution in the WG-based matrix but still the fibers exhibited a tendency to form clusters. Uneven distribution of natural fibers in protein matrices of different types has been reported in several previous studies [7, 14, 15] and this results in weaker, unreinforced zones in the material. The uneven fiber distribution in the present study was observed with SEM but also the relatively high standard deviation found during tensile testing indicated uneven distributions of fibers. In fact, the standard deviation decreased from 52 and 40% for, respectively, the Young's modulus and fracture strain, to 38.0 and 30.0% by switching from the electrical blender [14] to the high speed grinder. One reason for the remaining uneven distribution of fibers in the material prepared by the high speed grinder could be the small volume capacity of the grinder itself, leading to that larger samples had to be made from several small batches. However, fibers themselves are also known to exhibit a high variation in tensile properties. The properties of natural fibers are influenced by a number of factors including plant variety, climate variations, harvest times, maturity, retting, methods of decortication, and other technical processes [21]. Variations in these factors lead to large variation in plant fiber properties such as tensile strength and surface geometry [6]. Plant fibers exhibit many defects of various types contributing to the large non-uniformity. Structural defects relate to chemical

composition, crystallinity, cracks, and deformation faults. Geometrical defects relate to cross-sectional shape, diameter, and length [22]. This variation, shown in the present investigation as variation in fiber width with similar variation in all fiber classes, exists also among fibers from plants grown in the same plot, and even within groups of fibers from the same plant [23]. Of course, these drawbacks have to be considered together with the advantages (renewability, high strength and elastic modulus, low density, non-abrasiveness, and biodegradability [23]) when choosing fibers. Nevertheless, in the present study, similarly as has been found in a previous study [14] the fiber quality seemed to have relatively little influence on the tensile properties. A more proper distribution and bonding of the fibers to the protein matrix would most likely change the facts so that fiber quality would have played a role for the tensile properties of the WG-hemp composite material [14].

From the present study, it was obvious that the use of the high speed grinder did not improve the tensile properties as compared to using the electrical blender. Thus, other solutions are needed to obtain a more even distribution and better tensile properties. Suggestions include the use of sodium dodecyl sulphate, making the samples less viscous and/or to further decrease the fiber size into micro/nano scales. Also, the addition of alkali solutions to decrease the interfibrillar region by removing the hemicellulose and lignin might be one option [15], as well as using high-shear composite processing (e.g., twin-screw extrusion and injection molding [7, 15]).

Beside the need of better fiber distribution, there is also need of a better bonding between the hemp fiber and the protein matrix in natural composite materials [14, 24]. Different solutions have been suggested for better bonding, e.g., addition of compatibilizers, such as polyether amide grafted glycidyl methacrylate [7] or treatment of the fibers

with NaOH [24]. Another possible solution is the addition of a cross-linking agent, helping to create hydrogen bonds between the fibers and the plastic [14]. In the present investigation, the diamine was selected as one such possible cross-linker. However, neither with SEM nor with CLSM it was possible to detect any influence of diamine on the bonding between the fiber and the gluten matrix. Earlier studies showed that an increase in mechanical properties including tensile properties, flexural properties as well as impact strength was achieved by an addition of compatibilizer to soy-based bioplastics reinforced with fibers (pineapple leaf or grass fiber). Here the addition of a cross-linker had a positive effect on the tensile properties of the material (Young's modulus and maximum stress was increased with 36 and 25%, respectively, with the diamine). However, these effects were found to be mainly the result of an increased protein polymerization as observed with SE-HPLC and CLSM. Also, increase in tensile strength by addition of diamine to pure gluten indicated a stronger matrix protein network. In addition, fiber pull-outs suggested poor fiber–matrix bonding. Several studies have shown that a more polymerized gluten structure increases the tensile strength [4, 17, 25].

It is well-known that compression molded gluten films without any additives and/or any reinforcements have low tensile strength properties [17]. Earlier investigations have shown that addition of hemp fibers as reinforcement to WG films increases the tensile strength and Young's modulus considerably (doubled and tenfold, from 10–20 to 80–200 MPa) [12, 14]. Also, gluten composite materials, reinforced with other types of fibers, have been shown to double their tensile strength properties [9–11]. Other plant proteins as a base, e.g., soy proteins have in some investigations been found stronger as a base material as compared to those built on gluten [5, 7, 15]. So far, however, all types of biocomposites evaluated have far lower tensile strength properties compared to the best petroleum-based composite materials. Thus, to be able to use biocomposite materials to replace for present day's fiber composite materials as, e.g., glass or carbon fiber materials, there is an obvious need to improve the biocomposites. If one succeeds with that, a range of the biocomposites is clear, including car utensilities, building items, computer parts, etc. Of the different parameters used in the present study, it was obvious that a combination of adding diamine and using the highest amount of fibers, gave the highest stiffness (more than a sixfold increase in Young's modulus relative to materials without the two ingredients, from 23.7 to 149.6 MPa) and strength (maximum stress rose by more than 100%). If the main interest is improved strength/stiffness also other additives leading to increased protein polymerization could be of interest, including, e.g., NaOH [20]. It should be noted that with improved strength came

also poorer extensibility, especially at low fiber contents. To conclude, even though the diamine improved the strength/stiffness, we are still facing the challenges of finding the right route to optimal hemp fiber dispersion in the WGm and fiber/WG bonding. Optimal may, apart from improved mechanical properties, also mean that the amount of components used, their modifications, as well as the number of steps in the processing, have to be kept as small as possible. This is important from both a commercial and environmental view.

Conclusions

Use of the high speed grinder to blend the hemp fibers with the WGm led to a better fiber distribution compared to the use of an electrical blender. However, further improvement is needed as the fibers still exhibited tendencies to bundle together. The addition of the Jeffamine[®] polyetheramine (diamine) to the material did not seem to improve the bonding between the fibers and the matrix, but it improved the strength/stiffness of the WG matrix itself by an increased polymerization of the proteins. Further studies are required to find a way of improving the interaction between fibers and gluten plastic.

Acknowledgements This work was financed by Formas, Sweden. The authors thank Maria Luisa Prieto-Linde for practical help in the laboratory and Kerstin Brismar for her assistance with SEM.

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