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The impact of wounding and postharvest storage conditions on retention of soluble protein in sugar beet leaves

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Abstract: Sugar beet leaves can be a viable and economically interesting source of high-quality protein for the food industry. We investigated how storage conditions and leaf wounding at harvest affect the content and quality of the soluble protein. After collection, leaves were either stored intact or shredded to mimic wounding induced by commercial leaf harvesters. Leaf material was stored in small volumes at different temperatures to assess leaf physiology or in larger volumes to assess temperature development at different locations in the bins. Protein degradation was more pronounced at higher storage temperatures. Wounding accelerated the degradation of soluble protein at all temperatures. Both wounding and storage at higher temperatures greatly stimulated respiration activity and heat production. At temperatures below 5°C, ribulose-1,5-biphosphate carboxylase oxygenase (RuBisCO) in intact leaves was preserved for up to 3 weeks. At temperatures of 30–40°C, RuBisCO degradation occurred within 48 h. Degradation was more pronounced in shredded leaves. In 0.8-m^3 storage bins at ambient temperature, core temperatures rapidly increased, up to 25°C in intact leaves and up to 45°C in shredded leaves within 2–3 days. Immediate storage at 5°C greatly suppressed the temperature increase in intact but not in shredded leaves. The indirect effect of excessive wounding, that is, heat production, is discussed as the pivotal factor responsible for increased degradation of protein. For optimal retention of soluble protein levels and quality in harvested sugar beet leaves, it is advised to minimize wounding and to store the material at temperatures around −5°C.

KEYWORDS
protein, respiration, rubisco, storage temperature, sugar beet leaves, wounding

Practical Application: To preserve the soluble protein content and quality for at least 3 weeks, sugar beet leaves should be harvested with minimal wounding and stored at temperatures between 1 and 5°C. When aiming to store minimally wounded leaves in larger volumes, it must be ensured that the product temperature in the core of the biomass meets the temperature criterium or the cooling strategy must be adjusted. The principles of minimal wounding and low temperature storage are transferable to other leafy crops that are harvested for food protein.

1 | INTRODUCTION

Due to the increase in world population, a sustainable protein supply for food has become an important objective (Boland et al., 2013; Møller et al., 2021; Nieuwland et al., 2021). Currently, plant-based protein supply mainly comes from crops such as soybean, rapeseed, wheat, maize, and beet, but leafy crops, such as alfalfa, grasses, clover, and duckweed are considered useful alternatives (Møller et al., 2021; Nieuwland et al., 2021). As an agricultural sidestream, sugar beet leaves are also considered as an alternative protein source for the food industry (Martin et al., 2019; Møller et al., 2021; Tamayo Tenorio et al., 2017) and is currently being considered as a novel food by the European food safety authority (NF 2021/2370).

Sugar beets are mainly grown in the Northern hemisphere to produce crystalline sugar. During crop harvesting in autumn, the beets are harvested, while the leaves are left on the field as organic fertilizer (Tamayo Tenorio et al., 2017). These leaves contain valuable protein that is equivalent to about 400–600 kg ha^{-1} of protein, assuming an average of 22.8% crude protein on dry matter basis (Merodio & Sabater, 1988; Tamayo Tenorio et al., 2017). Of particular interest is the protein ribulose-1,5-biphosphate carboxylase oxygenase (RuBisCO), which is the most abundant protein in leaves of C_3 plants such as sugar beet and encompasses 20%–30% of leaf nitrogen and up to 50% of the soluble leaf protein (Evans & Seemann, 1989; Feller et al., 2008; Merodio & Sabater, 1988; Spreitzer & Salvucci, 2002). Compared to commercial egg white, whey, and soybean isolates, RuBisCO protein isolates from sugar beet leaves show comparable or even superior functional characteristics, that is, solubility, protein stability, gelling properties, and foaming capacity (Di Stefano et al., 2018; Martin et al., 2014, 2019; Møller et al., 2021). These characteristics highlight the potential of RuBisCO protein isolates as a sustainable alternative to animal (e.g., egg white and whey) or soy protein isolates and as a functional ingredient for a wide range of new applications in food (Di Stefano et al., 2018; Martin et al., 2014, 2019; Nieuwland et al., 2021). As such, over the last decade, various processing methods have been developed to extract leaf protein, particularly RuBisCO, which signifies the importance of making use of the leaves of agricultural crops such as sugar beet in the current protein transition (De Jong et al., 2014; Martin et al., 2019; Tamayo Tenorio et al., 2016).

While the chain for harvesting, storing, and processing sugar beet leaves to protein isolate has been investigated (Tamayo Tenorio et al., 2017), it has not yet been optimized. Particularly the effects of the steps between harvest and processing on the content, quality, and yield of protein are still unknown. For seasonal crops such as sugar beet leaves, the harvesting peaks necessitate considerable storage durations prior to central processing (Tamayo Tenorio et al., 2017). From this perspective, cooling and freezing have been suggested as the most suitable stabilization methods, but very limited data are available on the impact of temperature on the storage life and protein content of sugar beet leaves. Addition of compounds with antioxidative effects such as sulfite, metabisulfite, or polyvinylpolypyrrolidone during sugar beet leaf protein extraction highlights the sensitivity of the protein to, for example, polyphenol oxidation after leaf wounding (De Jong et al., 2014; Martin et al., 2014, 2019; Merodio & Sabater, 1988). Commercial beet harvester defoliation generally involves flail defoliation, which results in considerable wounding of the leaves and will likely negatively affect their storage life, with consequences for the quantity and quality of the leaf protein.

While the effects of cooling and wounding on sugar beet leaf quality are poorly documented, they have been extensively studied for fresh-cut produce, including leafy vegetables (Hodges & Toivonen, 2008). Minimal processing of leafy vegetables involves wounding and this can cause biological reactions such as browning, loss of green color, enhanced water loss, and an up to 300% increased respiration (Cantwell & Suslow, 2002; Degl'Innocenti et al., 2007; Deza-Durand & Petersen, 2011; Kim et al., 2005). These symptoms all become more apparent with increased temperature. While often unmentioned, the

increase in respiration and metabolism by itself will lead to increased heat production and further stimulation of respiration. As such, cooling is paramount to minimize these symptoms (Cantwell & Suslow, 2002; Hodges & Toivonen, 2008).

To support an efficient and sustainable chain for the extraction of sugar beet leaf protein, targeting high yield and quality, we have assessed how leaf wounding during harvest and postharvest cooling and freezing affects leaf protein in sugar beet leaves. First, we assessed how storage for up to 21 days at temperatures ranging from −5 to 20°C affects the RuBisCO content in intact sugar beet leaves. Second, we assessed how temperature and leaf wounding affect respiration, RuBisCO protein contents, and product temperature kinetics during storage.

2 | MATERIALS AND METHODS

2.1 | Plant material and storage conditions

Sugar beet (*Beta vulgaris* ssp. *vulgaris* cv. *altissima*) were grown in Wageningen, the Netherlands during 2019 and 2021, according to commercial Dutch growing protocols. For the storage duration experiment, leaves including the top part of the beet were harvested mid-October 2019, using sharp knives. After transport to the laboratory, leaves were excised from the beet, excluding the oldest and youngest leaves. From these leaves, sample bundles were prepared of five leaves each, which were tied together at the stem using rubber bands. These sample bundles were weighed and subsequently placed in plastic crates onto a thin polystyrene mat. Three sample bundles per crate were covered with three more bundles to restrict dehydration of the sample bundles and, in addition, the sides of the crates were wrapped in plastic to limit overall dehydration. In total, 24 plastic crates were divided over four temperatures (−5, 1, 10, and 20 ± 0.5°C in darkness). After 0, 1, 3, 7, 14, and 21 days, one crate per storage temperature was taken for sampling and further analysis.

For the leaf shredding and temperature experiment, leaves were harvested at the end of October 2021 using sharp knives, with exclusion of the oldest leaves. After harvest, leaves were collected in large plastic crates (0.8 m^3) under field conditions. Some of the crates had been equipped with temperature sensors at positions of interest (Figure 1). After transport of the crates to the laboratory, half of the leaves were shredded using a multi-shredder (BU-200, M.T. Johmann Landmaschinenbau, Limbach, Germany). The size of the leaf pieces after shredding was 5–30 cm^2. Leaves were shredded directly into crates equipped with temperature sensors. Sensor-equipped crates contained either 75 kg of intact leaves or 170 kg of shredded leaves. Crates were subsequently stored at 5 or 20°C, at 95% relative humidity, and in darkness, with one crate of intact and one crate of shredded leaves at each temperature. Leaves were stored for 6 days, while the temperature in the crates was monitored. After storage, leaf material was taken from the center-middle and center-bottom of the crates for further analysis.

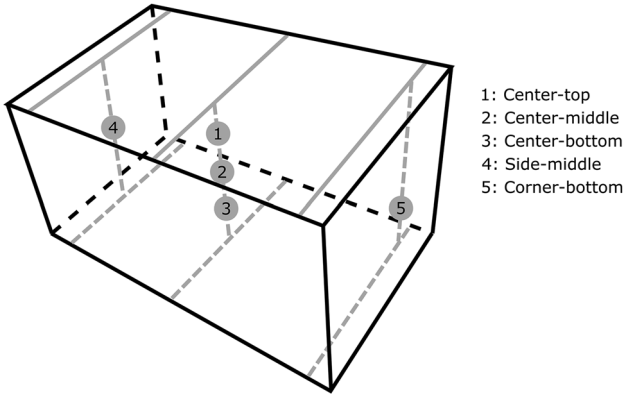

FIGURE 1 Location of temperature sensors within the bins.

In addition to storage in crates, samples of approximately 1 kg of intact and shredded leaf material were stored in open 10-L high-density polyethylene (HDPE) drums (Engels Logistiek B.V., Eindhoven, The Netherlands) and placed in triplicate at 5, 20, 30, and 45°C at 95% RH for up to 2 days. At several time points during storage, weight and respiration of the leaf material were measured. After 2 days, leaf material was taken from the center of the mass for color and protein analysis.

2.2 | Temperature

Temperature in the large crates was monitored using temperature probes (Pt-8316/S, range −40 to +60°C, Nootdorp, the Netherlands) connected to self-logging docking stations (custom ordered, TD-Studio, Wageningen, the Netherlands). In the center, viewed from the top of the bin, probes were placed near the top (1), middle (2), and bottom (3) of the bin (Figure 1). Additional probes were placed approximately 10 cm from the side at middle height (4) and in a corner, approximately 10 cm from the sides and bottom (5). Collected data were processed in Microsoft Excel.

2.3 | Respiration

To measure the respiration, the HDPE drums with leaf material were closed, after which oxygen (O_2) and carbon dioxide (CO_2) percentages were assessed immediately using a Checkpoint 2 (Dansensor A/S, Ringsted, Denmark)

via rubber septa (Suba-Seal, Sigma-Aldrich) mounted in the drum lids. After approximately 3 h of incubation, the measurement was repeated (at 45°C storage, incubation was ~ 1 h). CO_2 production and O_2 consumption were calculated using Equation (1):

$$\Delta n = \frac{\Delta V \times P}{R \times T_{\text{storage}}}, \quad (1)$$

where ΔV, P, R, and T_{storage} represent the change in O_2 and CO_2 in the sample headspace (L), the pressure (Pa), the gas constant (L Pa K^{-1} mol^{-1}), and the respective storage temperatures (K), respectively. The number of moles was subsequently normalized for sample weight (kg) and headspace accumulation time (s).

2.4 | Leaf color

Leaf material was distributed over blue serving trays from which color-calibrated images were obtained using an RGB camera (MAKO G-192C POE, Allied Vision, Stadtroda, Germany) positioned in an LED light cabinet (Designed by WFBR and build by IPSS Engineering, Wageningen, the Netherlands). Images were calibrated using a 24-patch color checker card (Color checker classic, X-rite Europe GmbH, Regensdorf, Switzerland). Intact leaves were imaged from both sides, since both sides visibly differed in color. Color analysis was done using multi-threshold color image segmentation in the RGB color space using a software tool developed at WFBR (Wageningen Food & Biobased Research, Wageningen, the Netherlands). Color values ($L^*a^*b^*$) from both sides of the leaves were averaged and color differences (ΔE) compared to fresh leaf values ($L_0{}^*a_0{}^*b_0{}^*$) were calculated according to Equation (2):

$$\Delta E = \sqrt{\left(L^* - L_0^*\right)^2 + \left(a^* - a_0^*\right)^2 + \left(b^* - b_0^*\right)^2}. \quad (2)$$

2.5 | Weight and dry matter percentage

Sample weights were recorded using an MS6002TS balance (Mettler-Toledo GmbH, Giessen, Germany). Dry matter percentage was determined by drying 100 g of leaf material in a hot-air oven at 80°C for 5 days and applying Equation (3):

$$\frac{\text{Dry weight}}{\text{Fresh weight}} \times 100\%. \quad (3)$$

2.6 | Protein extraction and analysis

Samples for protein extraction from intact leaves were prepared from five leaves of average size. The leaf material was quickly cut into 1-cm-wide strips, flash frozen in liquid nitrogen, put in 50 mL tubes, and stored at −80°C. Samples for protein extraction of shredded leaves were directly flash-frozen in liquid nitrogen, put in 50 mL tubes, and stored at −80°C. The following week, samples were ground using a liquid nitrogen precooled IKA® A11 analytical mill (IKA®-Werke GmbH & Co., Staufen, Germany), after which they were returned to −80°C until extraction.

To 100 mg of ground leaf material, 650 µL of AS08 300 extraction buffer for soluble protein (Agrisera AB, Vännäs, Sweden), pH 8.5, supplemented with proteinase inhibitors (Complete®; Roche, Basel, Switzerland) was added, and a 5-mm tungsten carbide bead (Qiagen) was also added. Samples were milled at 30 Hz for 2 min in an MM301 Vibration Mill (Retsch, Düsseldorf, Germany), after which the precooled tube holder (−20°C) was turned over and the sample milled once more for 2 min at 30 Hz. Following this, samples were centrifuged at 10,000 × g for 5 min, and the supernatant was carefully transferred to an Eppendorf tube, which then was centrifugated once more at 10,000 × g for 5 min. This final supernatant was transferred into new Eppendorf tubes and stored at −20°C until further analysis.

Protein contents were determined using a Pierce BCA protein assay. The soluble proteins in the supernatants were characterized by reduced SDS–polyacrylamide gel electrophoresis (SDS-PAGE).

For the storage duration experiment, thawed extracts were resuspended and diluted in 1:1 (v/v) ratio with loading buffer (2×) containing reducing agent (0.125 M Tris-HCL buffer pH 6.8 containing 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.04% bromophenol blue).

For the leaf shredding and temperature experiment, samples were diluted with loading buffer (1×) to a final concentration of 1 mg protein per milliliter.

The samples were subsequently heated for 10 min at 99°C in a shaking heating block and centrifuged at 12,000 × g for 5 min. An amount of 12.5 µL of each sample and 6 µL marker were loaded on a precast protein gel (Any kD Mini-Protean TGX gel, 12-well, #4569035). Electrophoresis was carried out at 180 V for about 1 h. Protein bands were stained with Coomassie Brilliant Blue (0.02% [w/v] Serva R250 in 50% methanol, 12% acetic acid) for 30 min at 35°C and de-stained overnight at

room temperature using a 10% methanol–10% acetic acid solution.

2.7 | Data analysis

Data were analyzed and graphs were prepared using Microsoft Excel. Statistical analyses (one-way and two-way ANOVA with Bonferroni post hoc tests) were done using Genstat 22 (VSN international Ltd, Hemel Hempstead, England, United Kingdom).

3 | RESULTS

3.1 | Effect of storage temperature on sugar beet leaf protein

Manually harvested sugar beet leaves were stored for up to 3 weeks at −5 to 20°C. Leaves at −5 and 20°C were visually wilted after 14 days, whereas leaves stored at 1 and 10°C were visually of good condition (Figure 2a). Dry matter percentage of leaves increased gradually over time and increased significantly after 14 days at −5 and 20°C (Figure 2b). The extracted soluble protein content, as determined by BCA and corrected for fresh weight, showed a pattern that was similar to that of the dry matter content (Figure S1). Since dehydration had been observed in these samples, we also determined the protein content on a dry weight basis. This showed that the protein content was relatively stable at −5, 1, and 10°C for 3 weeks and that at 20°C, the protein content had decreased significantly after 14 days (Figure 2c). Since protein contents determined by BCA do not distinguish between protein, protein fragments, peptides, and certain amino acids, we also studied the degradation of RuBisCO by SDS-PAGE. The intensity of the bands on the gels reflects the concentration of the protein or small protein fragments within the sampled leaf material, as samples were loaded without normalizing for protein content. The SDS-PAGE showed that storage at −5°C resulted in a relative increase in the band at 53 kDa, which in leaves mainly represents RuBisCO, whereas at 1°C, the RuBisCO content stayed constant throughout the 3 weeks of storage (Figure 2d). At 10°C, RuBisCO concentration remained constant for up to 14 days, but after 21 days RuBisCO concentration had decreased. At 20°C, RuBisCO concentration showed a decrease within 7 days of storage. Also here, the increased intensity in the lanes belonging to 14- and 21-day samples was likely due to a decreased moisture content in the samples due to leaf dehydration (Figure 2b,d).

Altogether, these results indicated that intact beet leaves harvested for protein extraction could be stored for up to at least 21 days at 1°C or at below zero temperatures, for up to 14 days at 10°C, and for up to 3 days at 20°C without protein degradation.

3.2 | Leaf wounding and temperature decreased storability of leaves for extraction of protein

Under practical conditions, the harvesting and handling of leaves may lead to excessive wounding and, in addition, more extreme storage temperatures in large bins with biomass may occur. To assess the effect of increased leaf wounding on the loss of protein, several additional experiments were done.

Smaller batches of intact and shredded leaves were stored in open HDPE drums at 5, 20, 30, and 45°C for 2 days. After 2 days, the green color of intact leaves was fully retained at 5, 20, and 30°C, whereas at 45°C the leaves turned dark brown with hints of purple (Figure 3a). This was also reflected in the color differences compared to the average L^*, a^*, and b^* values of fresh leaves (Figure 3b). Due to some color variation in the fresh leaf samples, color differences of fresh leaves compared to their average L^* a^* b^* values were included as well. Compared to the average color of fresh leaf samples at the start of the experiment, leaves did not significantly change color when they were stored for 2 days at temperatures up to 30°C, but color changed significantly after storage at 45°C. Shredded leaves remained relatively green after 2 days at 5°C, showed increased brown coloration at 20 and 30°C, and were dark brown with hints of purple at 45°C (Figure 3c). Compared to fresh shredded leaves, a small color difference was noticeable after storage at both 5 and 20°C. This difference was larger after storage at 30°C and considerably larger at 45°C.

During the 2 days of storage, weight loss increased steadily in both intact and shredded leaves, with increasing temperatures resulting in increased water loss, mainly visible in the leaf material on the top in the drums. After 2 days, intact leaves lost about 3% water at both 5 and 20°C, whereas at 30 and 45°C leaves lost 12% and 13%, respectively (Figure 4a). Shredded leaves lost increasing amounts of water with increasing temperatures: 2%, 6%, 12%, and 18% at 5, 20, 30, and 45°C, respectively (Figure 4b).

Oxygen consumption in intact leaves stored at 5 and 20°C started relatively low at 0.6 and 1 μmol kg^{-1} s^{-1} and over time gradually decreased to 0.4 and 0.8 μmol kg^{-1} s^{-1}, respectively (Figure 4c). In contrast, at 30 and

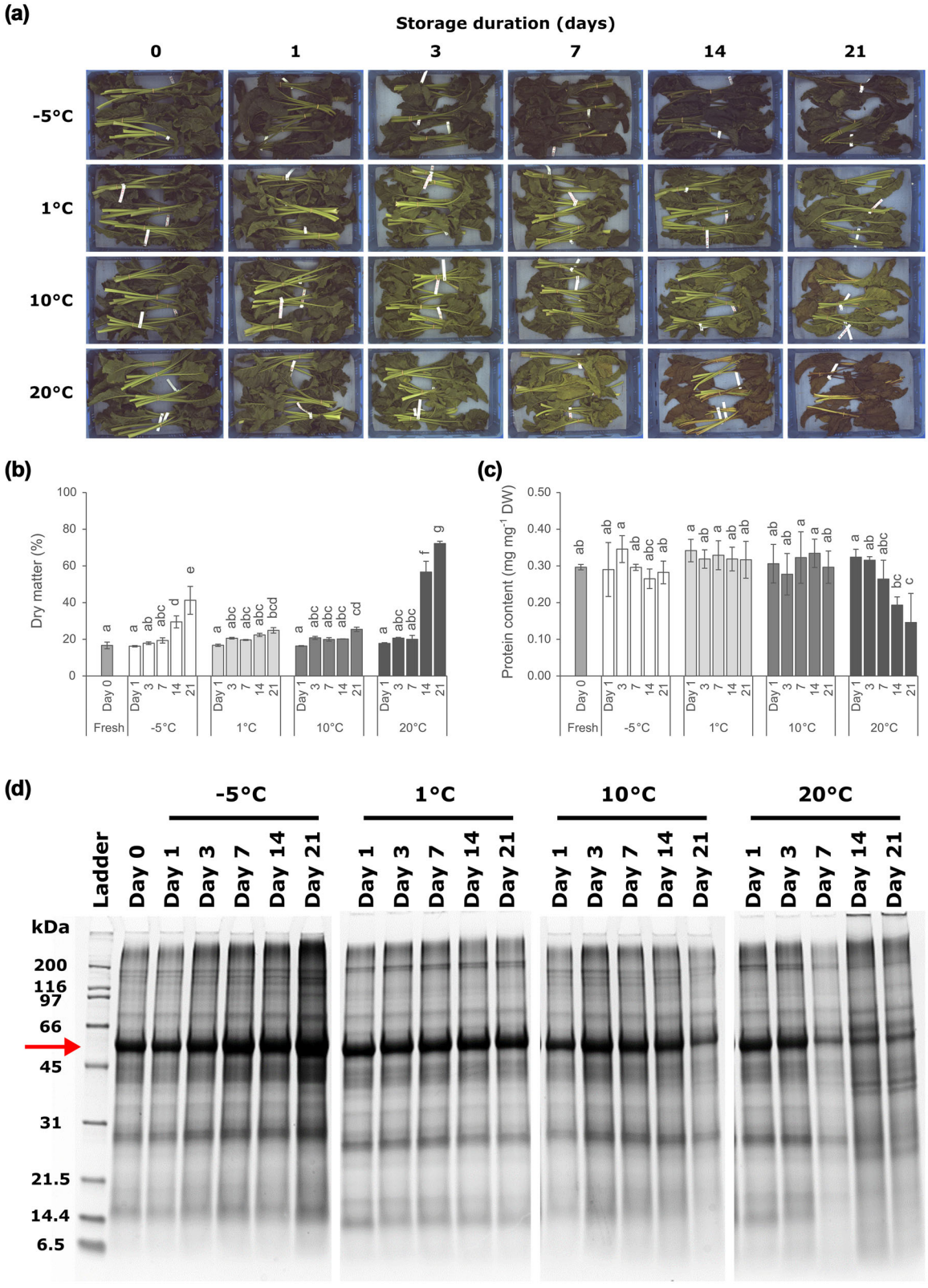

FIGURE 2 Effect of storage at different temperatures on manually harvested sugar beet leaves. Effects are shown on the appearance (a), dry matter (b), soluble protein content (c), and relative RuBisCO large subunit abundance (red arrow, 53 kDa) on SDS-PAGE gels (d). Equal volumes of protein extract were loaded on the gels. Leaves were either fresh (day 0) or were stored for 1, 3, 7, 14, or 21 days at −5, 1, 10, or 20°C. Data represent means ± 95% CI, $n = 3$. Statistical notations represent groups with significant differences (two-way ANOVA with Bonferroni post hoc test, $\alpha = 0.05$).

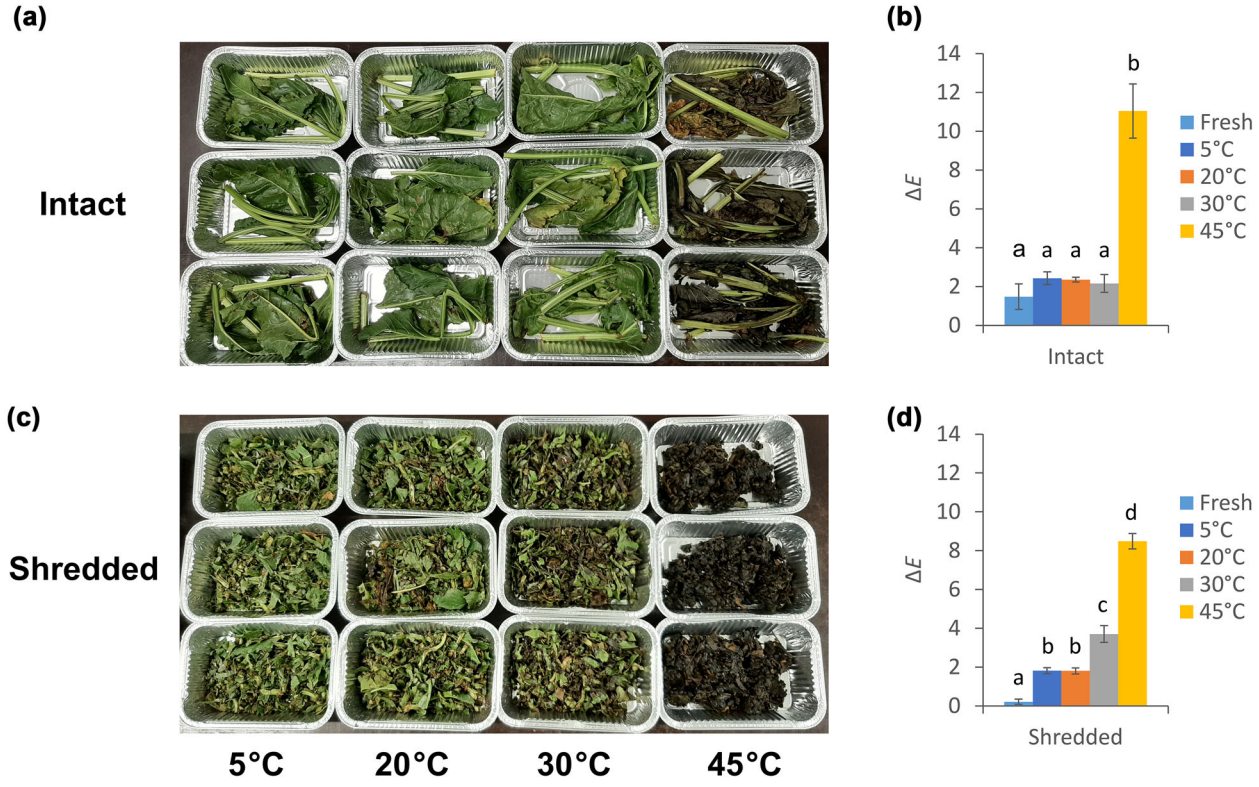

FIGURE 3 Appearance and color development of leaf material stored in HDPE drums at different temperatures for 2 days. Leaf material, intact or shredded, was stored at 5, 20, 30, and 45°C (a). Color differences (ΔE, CIELab) between the fresh leaf average and leaves stored at 5, 20, 30, and 45°C are shown for cut (b) and shredded leaves (c). Data are shown as means ± 95% confidence intervals ($n = 3$). Statistical notations represent groups with significant differences (ANOVA with Bonferroni post hoc test, $p < 0.05$).

45°C, O_2 consumption steadily increased from 1 and 2 to 2 and 6 μmol kg^{-1} s^{-1}, respectively. In shredded leaves, O_2 consumption was initially at a higher level than in intact leaves. Oxygen consumption in shredded leaf material stored at 5°C gradually decreased, whereas storage at higher temperatures caused threefold increases during the first 24 h, after which the O_2 consumption rate gradually decreased to twofold the initial rate (Figure 4d).

CO_2 production followed similar patterns as the O_2 consumption (Figure 4e,f), although CO_2 production in shredded leaves at 30°C decreased a bit more after their initial increase and CO_2 production in shredded leaves at 45°C showed a sharper increase and consecutive decrease (Figure 4f).

Overall, respiration of shredded leaf material was up to three times higher than respiration of intact leaves, which also implies that shredded leaf material produces up to three times as much heat.

During the 2 days of storage, the soluble protein content in intact leaves, as determined with BCA, showed no significant decrease, irrespective of the storage temperatures (Figure 5a), whereas the protein content in shredded leaf material decreased during storage at 30 and 45°C (Figure 5b). Since BCA protein content measurements may also detect already degraded protein, we also included an SDS-PAGE analysis. The gel was loaded with equal amounts of total soluble protein to inspect the RuBisCO large subunit. The 53 kDa band representing the RuBisCO large subunit was clearly visible in extracts from intact leaves that were fresh or stored for 2 days at 5 or 20°C (Figure 5c). However, extracts from leaves that were stored at 30 and 45°C showed this band at decreased intensities. In extracts from shredded leaves that were either fresh or stored for 2 days at 5°C, the 53-kDa band was less intense than in extracts from fresh intact leaves. This indicates that the shredding by itself already initiates protein breakdown. In extracts from shredded leaves stored for 2 days at 20, 30, or 45°C, the 53-kDa band was completely absent.

Under practical circumstances, leaf material is likely to be collected in larger storage bins for transport and storage, compared to the experimental settings described so far in this work. To see how the temperature of larger collections of leaf material develops over the course of several days under different storage conditions, we used temperature probes to monitor the temperature development at different positions in 0.8-m^3 storage bins filled with either intact or shredded leaf material. The packing densities of intact and shredded leaf material in the bins were 94 and

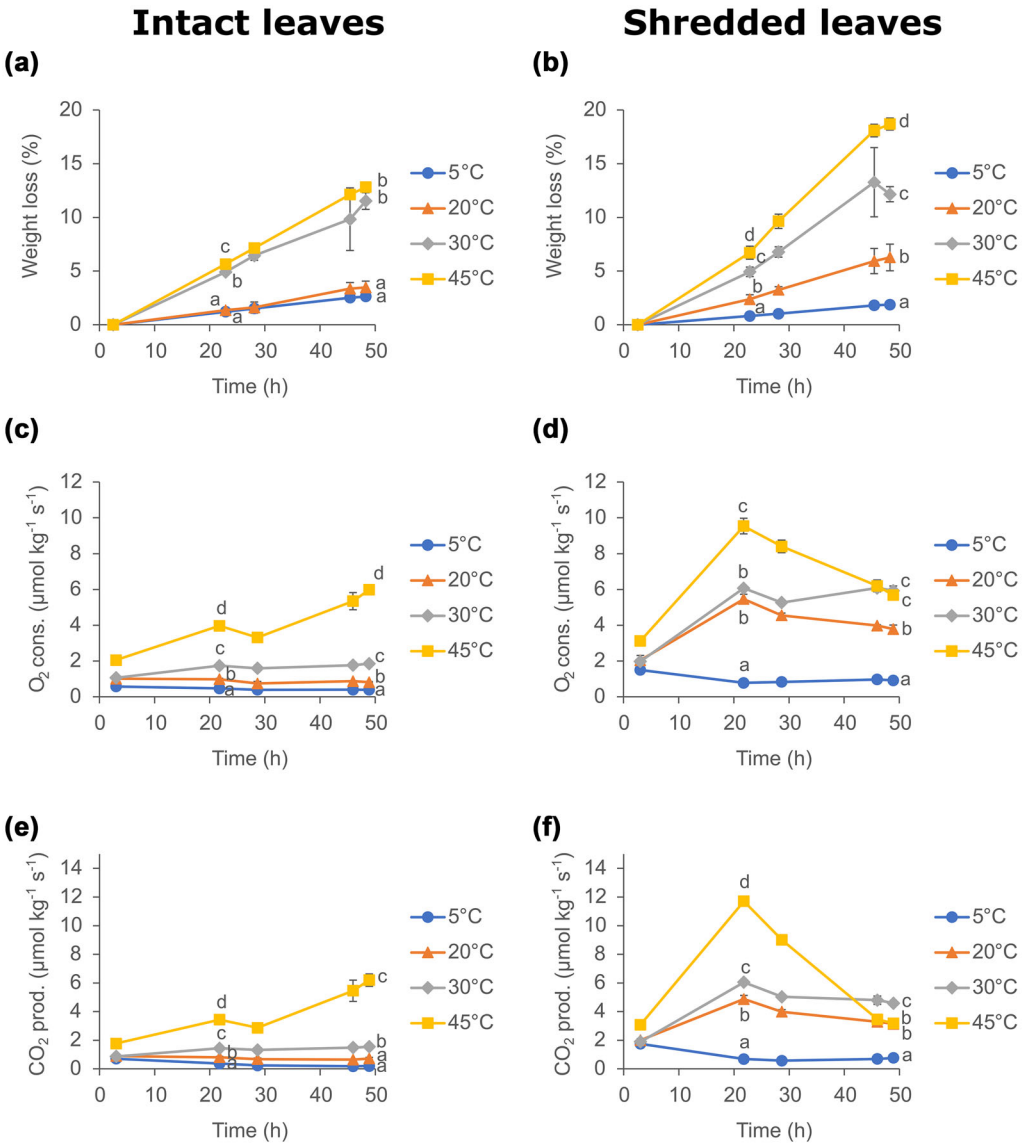

FIGURE 4 Dehydration and respiration of leaf material stored in HDPE drums at different temperatures over 2 days. Dehydration was measured as weight loss (a and b) and respiration was assessed by O_2 consumption (c and d) and CO_2 production (e and f). Leaf material, intact (a, c, and e) or shredded (b, d, and f), was stored at 5, 20, 30, and 45°C. Data are shown as means ± 95% confidence intervals ($n = 3$). Statistical notations represent groups with significant differences after 24 and 48 h (ANOVA with Bonferroni post test, $p < 0.05$).

212 kg m^{-3}, respectively. For intact leaves stored at 5°C for 6 days, the temperature in the bin was maintained at about 5°C and there were no differences in temperature between the different probe positions (Figure 6a). For shredded leaves stored at 5°C, significant increases in temperature were observed, up to 47°C near the center-middle and center-top of the bins and up to 40°C near the side-middle of the bin (Figure 6b). The maximum temperatures at the different positions were reached between 2.5 (center-top and -middle) and 3.5 days (side-middle) of storage. After reaching its maximum, the temperature steadily decreased until the experiment was terminated after 6 days. For intact leaves stored at 20°C, a steady increase in temperatures up to 24°C was observed within 2 days, after which the temperatures did not change much (Figure 5c). The center-top and center-middle probes showed very similar patterns that rose and fell between 20 and 26°C. These probes had shifted their relative position in the leaf material as the leaf material settled and had ended up at the same height after the 6 days. During the settling, the probes seemed to have alternating exposure to climate room conditions (20°C) and the leaf material (26°C). Nevertheless, the temperatures around 26°C suggested a similar pattern as the side-middle probe, but at a slightly higher temperature. In general, the profile was similar for all positions and no great differences between positions were apparent. For shredded leaves stored at 20°C, a rise in temperature was observed at all probe positions, with peak values up to

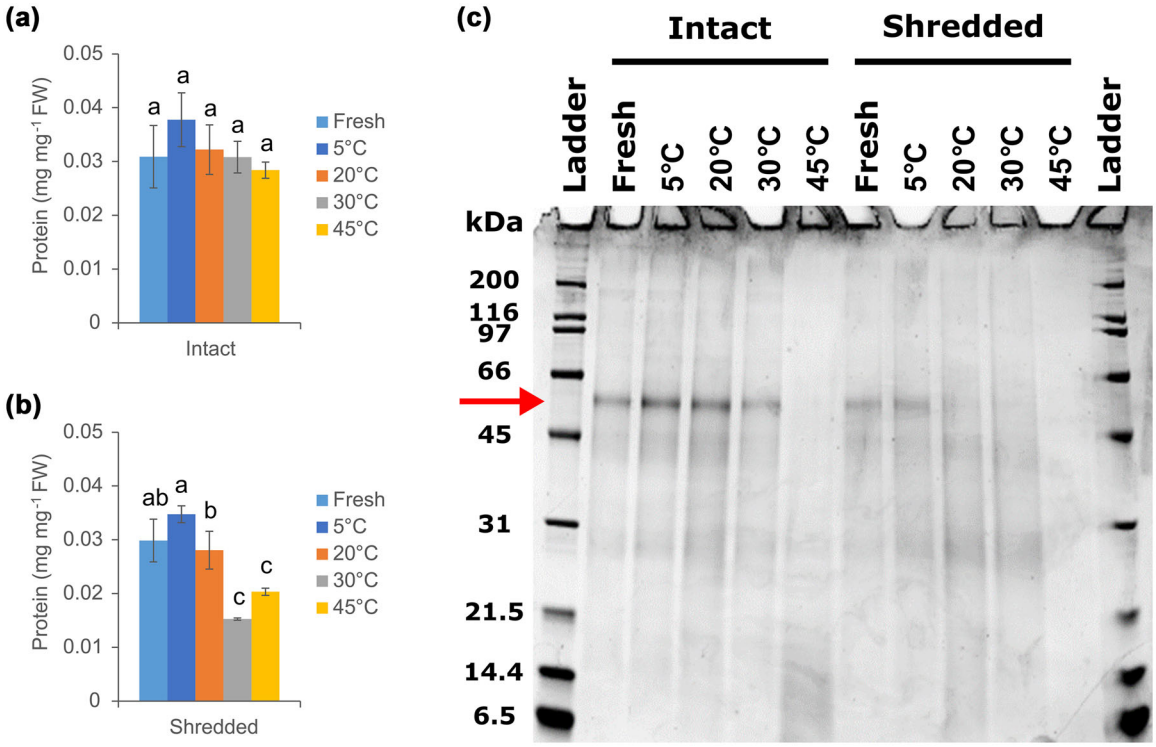

FIGURE 5 Protein degradation in leaf material stored in HDPE drums at different temperatures for 2 days. Protein degradation is shown as soluble protein content in intact (a) and shredded leaves (b) and by relative RuBisCO large subunit abundance (red arrow, 53 kDa) on an SDS-PAGE gel (c). Equal amounts of protein were loaded on the gel. Leaf material was stored at 5, 20, 30, and 45°C. Data are shown as means ± 95% confidence intervals ($n = 3$). Statistical notations represent groups with significant differences (ANOVA with Bonferroni post hoc test, $p < 0.05$).

41°C. Peak values were reached soonest at the top and middle positions of the bin (Figure 5d). The top probe showed a sharp decline after about 1 day, which stabilized around 23°C after 2 days. This suggests that the probe became exposed to the climate room conditions due to settling of the leaf material. For the middle and bottom probes, once the peaks were reached, temperatures steadily declined to 27 and 24°C, respectively.

During sampling of the leaf material, temperatures were notably higher in the middle compared to the bottom of the bins, particularly for the shredded leaves. In the case of shredded leaves at 5°C, we could even observe that the leaf material at the sides of the bin was still green, whereas it was brown at the center of the bin (Figure S2a). Furthermore, shredded leaf material from the center of the bins stored at 5 and 20°C smelled of silage, with the smell being less obvious at 20°C. Samples for analyses were taken from the center-middle and center-bottom of the bins and were used to determine the soluble protein content and for SDS-PAGE analysis.

At both sample positions, the soluble protein content was generally higher in leaf extracts from intact leaves compared to shredded leaves. In addition, the soluble protein content decreased as a result of storage and this decrease was more pronounced following storage at 20°C than at 5°C. These results confirmed the earlier observations.

For intact leaves, under both storage conditions, there were no differences in temperature between the sample positions. This was reflected by similar soluble protein levels in the samples from both positions. For shredded leaves, the different sample positions had different temperature histories. At 5°C, the bottom position was on average about 12–13°C, whereas the middle position reached a peak of 45°C (Figure 6b). This, however, was not reflected in the soluble protein content levels that were approximately similar (Figure 7a,b). At 20°C, shredded leaf material at the middle position clearly experienced a higher temperature than material at the bottom position (Figure 6d). This was indeed reflected by a lower soluble protein content in samples from the middle position (Figure 7a,b).

SDS-PAGE analyses showed that in intact leaves, RuBisCO was preserved during storage for 6 days at 5°C but fully degraded at 20°C. For shredded leaves, both after storage at 5 or 20°C, RuBisCO was fully degraded (Figure 7c,d). There were no clear differences in RuBisCO between the different sample positions center-middle and center-bottom.

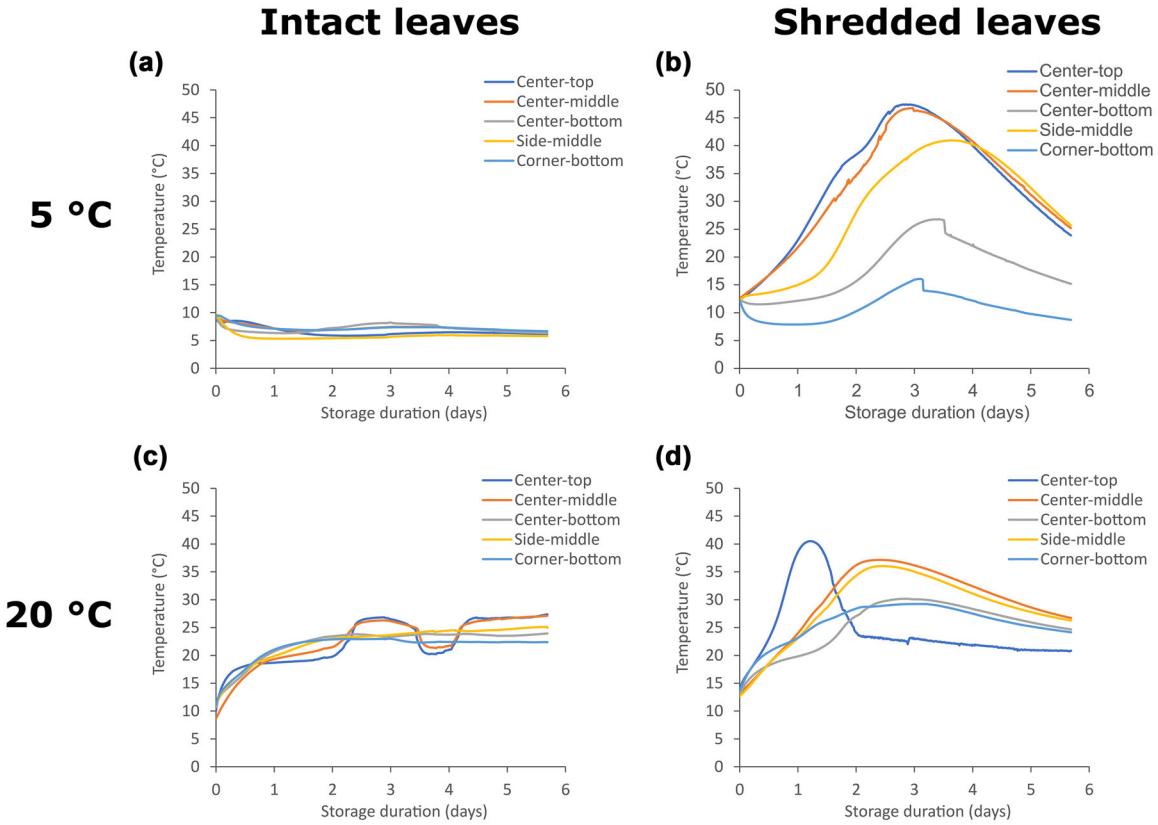

FIGURE 6 Temperature development in bins with leaf material stored for 6 days. Bins were stored at either 5 (a and b) or 20°C (c and d) and contained either 75 kg of intact (a and c) or 170 kg of shredded leaf material (b and d). Probes were placed either in the center-top, center-middle, center-bottom, side-middle, or corner-bottom. Data were logged every 15 min.

4 | DISCUSSION

4.1 | Low storage temperatures reduce degradation of RuBisCO in intact sugar beet leaves

In postharvest research, it is well accepted that reduced temperature maintains the quality of leafy vegetables (Cantwell & Suslow, 2002; Hodges & Toivonen, 2008). As such, it was to be expected that storing sugar beet leaves at low, nonfreezing temperatures maintained the leaf quality (Figure 2a). The new information achieved here was that RuBisCO integrity was maintained for up to 3 weeks at 1°C and up to 2 weeks at 10°C (Figure 2d), illustrating that it is indeed feasible to store sugar beet leaves and still be able to extract RuBisCO protein.

The experiment also showed that at more extreme temperatures (−5 and 20°C), the dry matter and total protein contents on fresh weight basis had strongly increased (Figures 2b and S1), which coincided with the wilting observed in the leaves (Figure 2a). This relative increase in dry matter and protein was related to excessive water loss of the leaves, which amounted up to 50% at 20°C. The water loss was caused by a lack of moisture regulation in the used storage cell.

Dark color development of the leaves stored at −5°C indicated leakage of cellular fluids into the apoplast, likely due to ice crystals penetrating the cell membranes (Palta & Weiss, 2018). Such leakage causes an increase of free water in the leaf material that can evaporate relatively easily. Plotting the protein content on a dry weight basis (Figure 2c) confirmed that the increase in total protein content was caused by the dehydration of the leaves and that storage at 20°C resulted in a decreased total protein content after 2 weeks.

Storage in smaller batches for 2 days and including moisture regulation in the storage cell greatly limited the evaporation of water from the leaves stored at 0 and 20°C (Figure 4a). However, intact leaves at 30 and 45°C still showed a markedly increased water loss presumably related to the vapor pressure difference between the atmosphere and the products internal air spaces. We are uncertain why this difference was so marked, but we suspect the higher temperatures to progressively trigger leaf responses, such as transpiration to cool down the leaves.

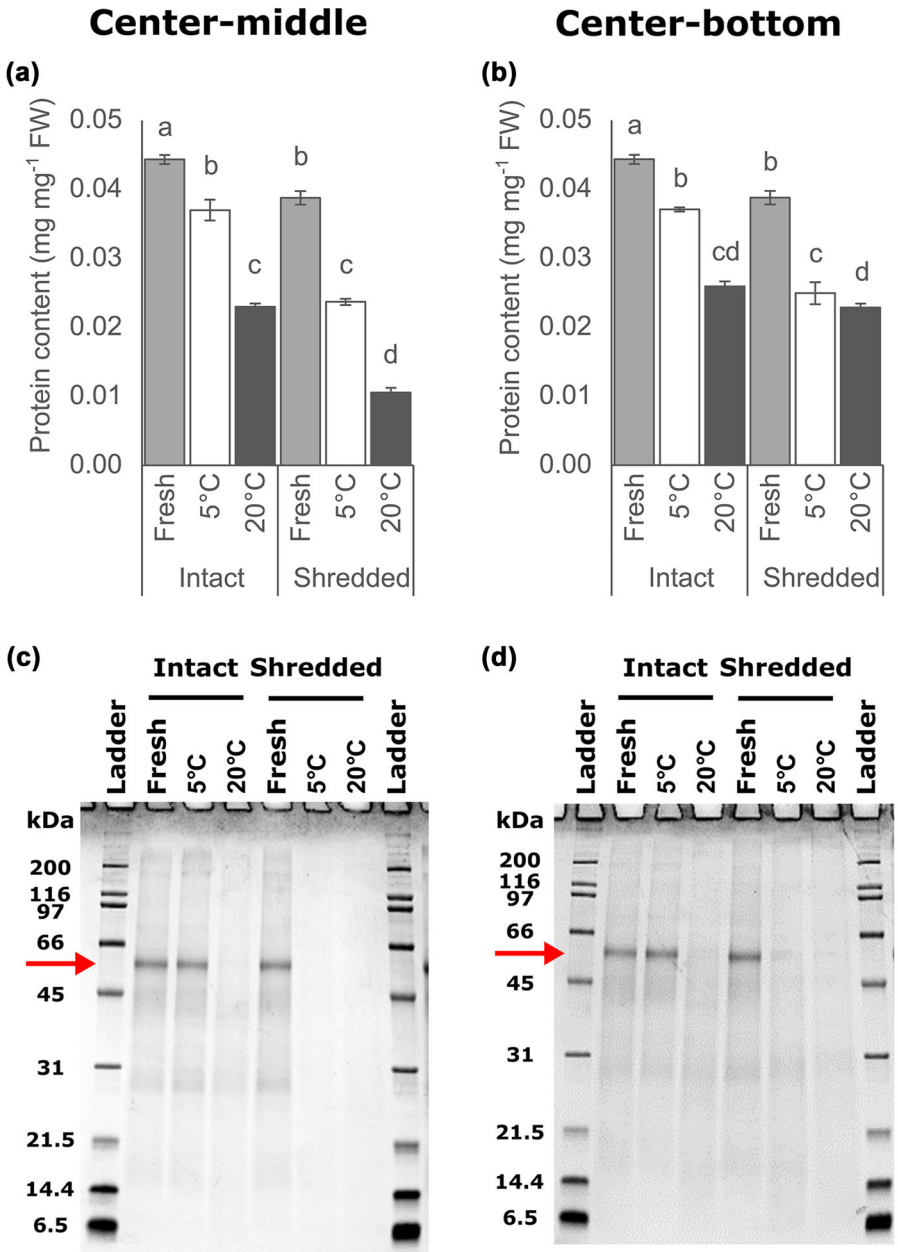

FIGURE 7 Protein degradation in leaf material stored in bins at 5 or 20°C for 6 days. Protein degradation is shown as soluble protein content (a and b) and by relative RuBisCO large subunit abundance (53 kDa) on an SDS-PAGE gel (c and d). Leaf material was intact or shredded and taken from either the center-middle (a and c) or center-bottom of the bin (b and d). Equal amounts of protein were loaded on the gel. The red arrow represents the RuBisCO large subunit (53 kDa). Data are shown as means ± 95% confidence intervals ($n = 3$ technical replicates). Statistical notations represent groups with significant differences (two-way ANOVA with Bonferroni post hoc test, $p < 0.05$).

Progressive damage related to temperature was also visible in the RuBisCO large subunit bands, which decreased in intensity at 30°C and were completely absent at 45°C (Figure 5c). Storage in larger batches for 6 days showed temperatures in the center of the batch to increase toward 30°C. This, combined with the longer storage time, was sufficient to completely degrade the RuBisCO in the leaves. Leaves stored in larger batches at 5°C remained rather stable in temperature (Figure 6a) and showed no degradation of RuBisCO protein (Figure 7c).

While freezing is an option that technically retained the protein (Figure 1d), it does cause cell leakage (Palta & Weiss, 2018). Upon defrosting, this will lead to mixture of subcellular compartments and increased polyphenol oxidase activity and protein losses that should be accounted for by including antioxidants

(De Jong et al., 2014; Martin et al., 2014, 2019; Merodio & Sabater, 1988).

4.2 | Wounding increased respiration, heat production, and protein degradation in sugar beet leaves

Wounding of sugar beet leaves due to shredding increased weight loss (only in leaves on top of the storage bins), respiration, browning, and loss of protein, including RuBisCO (Figures 3, 4, 5). Weight loss was likely increased due to the enhanced leaf surface of the shredded leaves, as well as the wounding "freeing" water from the cells that more easily evaporates. Respiration of both intact and shredded leaf material was reduced at lower temperature (Figure 4c–f). Wounding, here induced by shredding the leaf material, increased the respiration activity by over three times, similar to shredding of lettuce and cabbage (Cantwell & Suslow, 2002).

The effects of temperature and wounding on respiration of minimally processed (fresh-cut) vegetables such as lettuce, endive, or brassica species are well known (Cantwell & Suslow, 2002; Deza-Durand & Petersen, 2011; Martínez et al., 2005; Martínez-Sánchez et al., 2008). High respiration is associated with more rapid nutrient depletion and tissue senescence (Hodges & Toivonen, 2008; Iakimova & Woltering, 2018; Kim et al., 2005). For that reason, fresh horticultural products and especially fresh-cut products are stored at low temperatures (Cantwell & Suslow, 2002; Hodges & Toivonen, 2008). However, the effectiveness of a low storage temperature depends on how the heat, which is produced accompanying the respiration of the leaf material, is removed from the leaf mass. In our case, storage of intact (nonwounded) leaf material stored in larger bins at ambient temperature (20°C) led to an increase in biomass temperature up to 25–27°C after 2.5 days (Figure 6c). Cold storage at 5°C led to a maximum biomass temperature up to 7–8°C, illustrating the effectiveness of cooling (Figure 6a). Shredding of the leaf material introduced excessive wounding, a more compact packing (about 2.5 times the amount of biomass in the storage bin), and a greatly increased respiration (Figure 4c–f), which together led to an increased biomass temperature of up to 40–45°C in 3 days (Figure 6b,d). While the presence of microbial activity may have contributed to this increase in temperature, no visual and olfactory microbial activity was observed after 2 days at 20, 30, or 45°C in small bins, whereas the shredded leaf material at 45°C looked similar to that at the center of the large storage bin after 6 days of storage (Figures 3c and S2a). This suggests that, after 2 days, microorganisms were not present in sufficient quantity to result in silage, while the browning of shredded leaf material and associated loss of RuBisCO protein can be caused by 45°C within 2 days. Our observations therefore suggest that the increase in temperature during at least the first 2 days is caused mainly by physiological processes (e.g., respiration) within the leaf material. After this time, microorganisms involved in silage are likely to contribute, particularly since the degradation of the leaf material would make the nutrients easily accessible for microorganisms.

Cooling the shredded material did not greatly limit the increase in temperature in the center and top of the bins, likely due to insufficient heat exchange between the compact biomass and the air in the cooling room. This indicates that, even independent of the outside temperature, the core temperature of shredded biomass, in bins of 0.8 m^3, may quickly reach 40°C or more. Interestingly, the leaf material temperature did not exceed 40–45°C, which implies that the leaf material may be dying around this temperature. Indeed, leaf material at 45°C showed a strong decrease in respiration after 24 h (Figure 4d,f) and significant browning within 48 h (Figure 3a,c), which are generally associated with cell damage and oxidation (Cantwell & Suslow, 2002; Degl'Innocenti et al., 2007).

Browning of leaf tissue occurs when the enzyme polyphenol oxidase (PPO) converts *o*-diphenols, which are produced during leaf stress, into *o*-quinones that can form complexes with pinkish and brownish colors (Boeckx et al., 2017; Keppler et al., 2020; Saltveit, 2018). In intact leaves, PPO resides in plastids and *o*-diphenols reside in the vacuole, but when the two compartments are disrupted, for example, during wounding or senescence, *o*-quinones are formed. Besides forming pink- and brown-colored complexes, *o*-quinones can also aggregate with proteins, thereby affecting the functional properties of these proteins and their abundance in the protein isolates (Keppler et al., 2020; Kroll et al., 2003). As such, leaf browning is not desired when the goal is to acquire high-quality functional protein. Indeed, at the higher storage temperatures, browning (Figure 3) was always accompanied by dramatic reductions in RuBisCO (Figure 5c). Although tissue browning may contribute to protein degradation, in this case it is not the only cause, as RuBisCO abundance already decreased at temperatures where no detectable color differences were observed (Figure 3b,d).

The protein content (as measured by BCA method) in leaf material stored for 2 days seemed unaffected in intact leaves (Figure 5a) and decreased 30%–50% in shredded leaf material at 30 and 45°C (Figure 5c). However, within these 2 days of storage, severe RuBisCO degradation was already observed on SDS gels in intact leaves at 30 and 45°C and in shredded leaf material also at 20°C. This apparent discrepancy between the two observations can be explained by looking more closely at both methods used

to determine protein. In complete plant systems, protein degradation products are relocated to sink tissues and disappear relatively quickly, whereas in absence of active sink tissues, such as in detached leaves and darkened plant systems, the protein degradation products are retained in the leaf tissues (Law et al., 2018). As a result, the leaf material becomes significantly enriched in free amino acids, including tryptophan and tyrosine (Law et al., 2018). The BCA method reacts with the amino acids—cysteine, tryptophan, and tyrosine—as well as with peptides, protein fragments, and proteins that contain these amino acids in their side chains (Olson & Markwell, 2007; Wiechelman et al., 1988). This implies that the protein contents presented in this work are better read in the context of total nitrogen, whereas the RuBisCO protein abundance on the gels likely reflects the soluble intact protein content. From this perspective, cooling of intact leaves greatly prevented protein degradation, allowing storage times of multiple weeks at temperatures between 0 and 5°C without significant loss of protein content or quality (Figure 2d).

Whether cold storage of sugar beet leaves will be a cost-efficient practice will depend on different factors, such as leaf damage during harvest, starting temperature of the harvested leaf material, method of cooling down and maintaining temperature during storage, centralized or decentralized storage and processing, required storage duration, processing efficiency of extracting protein from stored leaves, and market price of the extracted protein, among others (Tamayo Tenorio et al., 2017). Preliminary results on juice pressing sugar beet leaf material after storage in 0.8-m^3 bins indicated that juice yield of intact leaves stored for 6 days at 5°C was similar to fresh leaves and slightly less efficient with shredded leaves and intact leaves stored for 6 days at 20°C (Figure S2b). Furthermore, pressing and applied coagulation steps (De Jong et al., 2014) appeared to result in reduced protein contents in response to shredding, storage, and increased storage temperature (Figure S2c).

Storage of leaf material in larger bins confirmed the negative effects of both increased temperature and excessive wounding on protein degradation. Both the wounding and its indirect effect, heat production, are considered as the responsible factors for the increased degradation of protein during storage of shredded biomass.

Removal of heat from shredded leaf material is more difficult than that from intact leaves because the shredded material is more densely packed and exhibits more resistance to heat exchange. As such, it is hard to control the temperature of such biomass using a regular cold room. Furthermore, increasing storage bin sizes may lead to increased temperatures inside the bins, even with intact leaf tissues. In such cases, alternative cooling principles may be applied to control the temperature in the biomass, for example, forced air cooling principles that are used during precooling of fruit and vegetables after harvest (Thompson et al., 2008).

5 | CONCLUSIONS

We investigated the impact of wounding, for example, shredding, and storage temperature on the retention of protein in harvested beet leaves destined for protein extraction. In both intact and shredded leaf material, protein degradation was more pronounced at higher temperatures. Wounding further accelerated the protein degradation at all temperatures. Both higher temperatures and wounding stimulated the respiration activity and the associated heat production of the material. In larger volumes, that is, 0.8 m^3, the temperature within the biomass rapidly increased as a result of the heat production by the product, up to 25°C in intact leaves and up to 45°C in shredded leaves within 3 days. Placing 0.8-m^3 bins with intact leaves in a regular cooling room at 5°C greatly suppressed the heating up of the biomass and preserved the protein. Placing leaves at −5°C also preserved the protein, but causes frost plasmolysis and will likely lead to increased polyphenol oxidase activity during defrosting prior to protein extraction, which will have to be accounted for using antioxidants. The compact packing and high level of heat production of shredded material made it hard to control the temperature of the biomass.

To preserve the soluble protein content and quality for at least 3 weeks, sugar beet leaves should be harvested with minimal wounding and stored at temperatures between 1 and 5°C. When aiming to store larger volumes, it is important to further investigate the use of more effective cooling methods to maintain a low product temperature in the core of the biomass and efficiently preserve protein quality. The knowledge presented in this work should provide more flexibility in the processing of sugar beet leaf material for protein extraction, either on-site or at processing plants. Furthermore, the principles illustrated in this paper are transferable to other leafy crops considered as alternative sources of protein for food.

AUTHOR CONTRIBUTIONS

Bastiaan Brouwer: Conceptualization; methodology; validation; formal analysis; investigation; writing – review and editing; writing – original draft; visualization; data curation. **Maxence J. M. Paillart**: Conceptualization; methodology; validation; formal analysis; investigation; data curation; writing – review and editing. **Marieke E. Bruins**: Conceptualization; writing – review and

editing. **Edo Wissink**: Methodology; formal analysis. **Mariska Nijenhuis-de Vries**: Methodology; formal analysis. **Manon Mensink**: Methodology; formal analysis. **Helene Bode-Mocking**: Methodology; formal analysis. **Willemijn Liese**: Methodology; formal analysis. **Peter Geerdink**: Conceptualization; writing – review and editing. **Esther M. Hogeveen-van Echtelt**: Writing – review and editing; conceptualization; project administration; methodology; funding acquisition. **Ernst J. Woltering**: Writing – review and editing; supervision.

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CONFLICTS OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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