Betalain extraction from *Hylocereus polyrhizus* for natural food coloring purposes

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Abstract

Coloring preparations from *Hylocereus polyrhizus* have recently received attention because peel and/or aril of the fruit exhibit a high content of betacyanins. These pigments are of special interest due to their potential as a red food colorants with a high stability at neutral pH. To improve production yield and to obtain a concentrated *Hylocereus* fruit extract, Pectinex Ultra SP–L in various dosages was applied to degrade the mucilage and make seed removal easier. Analytical methods were used to assess characteristic quality parameters of the treated samples against a control. Colour stability and overall betacyanin retention was assessed to monitor pigment retention. Moreover, individual betacyanin stability for each sample was monitored by comparing peak areas of the respective pigments. Betanin, phyllocactin, hylocerenin, and their respective C_{15} –isomers were identified as the major betacyanin components in treated samplesInterestingly, these isomers appeared to be indicative of enzymation, while the control showed rather little contents. In addition, betanin and isobetanin presented best stability in all treatments whereas phyllocactin degraded fastest as reflected in lower values for the phyllocactin isomerization index.

Keywords: *Hylocereus polyrhizus;* betacyanins; natural red food colorant; enzymatic treatment; concentrated extract.

Introduction

Coloring foodstuffs have been put forward in recent years since their application does not require E–number declaration in Europe thus allowing clean labeling of colored foods (Stintzing and Carle, 2004). Furthermore, colorants derived from vegetables and fruits may impart nutritional value to the colored food item. In addition, there is scientific proof demonstrating that isolated pigments do not provide the same benefits as the consumption of foods with whole fruit or vegetable preparations (Konczak and Wei, 2004; Tesoriere *et al.,* 2004). Moreover, synergistic factors within the food matrix may promote bioavailability of naturally occurring food colors and therefore increase their efficacy (Stahl and Sies, 1999; Bitsch *et al.*, 2004).

In modern food technology, enzymes are used to hydrolyze high–molecular–weight substances such as pectin, protein, etc. thus improving extraction of valuable components contributing to color, texture or flavor. Since the presence of pectin is not desirable upon concentration of the coloring preparations due to increasing viscosity, pectic enzymes are typically applied as natural macerating

tool to improve yield, clarification, and filtration (Yadav *et al.,* 2009). Since *Hylocereus* is rich in mucilaginous substances counteracting processing, a strategy to degrade these high molecular weight substances and at the same time minimizing color loss is of utmost importance for future commercialization of pitaya–derived pigment preparations (Herbach *et al.,* 2007). Although a range of enzymes have been tested recently, the required dosage was not viable from an economic point of view (Herbach *et al.,* 2007). Therefore, a further study appeared to be of interest. In order to monitor structural changes of betacyanins, HPLC–UV analysis was performed, comparing peak areas of the characteristic betacyanins throughout the process. Since the fruit matrix has previously been reported to improve betacyanin stability (Herbach *et al.,* 2006a), the degree of pectin breakdown by application of different dosages may help to better understand pigment–matrix interaction.

Materials and methods

Plant material

Fruits of 2–year–old plants of *Hylocereus polyrhizus* (red skin, purple flesh) deposited and grown on a trellis system at a modern agricultural farm directed by Ministry of *Agriculture* & Agro–based Industry (*MOA*) in Johor, Malaysia, were used in this investigation. The sampled plants were originally brought from Taiwan as stems several years ago for *H. polyrhizus* plantation. Fruits were harvested for analysis when reaching full ripeness, 30–35 days after pollination. All analyses were performed on 10 random samples. The edible portion was separated from the skin with a stainless steel knife and the edible part of the fruits (pulp) was instantaneously smashed into ice for cooling. The homogenized pulp was adjusted to pH 4 by adding food–grade citric acid (Fluka Chemicals, New York, NY, USA) before further extraction.

Chemicals and standards

All reagents and solvents were purchased from Merck (Darmstadt, Germany) and Sigma–Aldrich (Selangor D.E., Malaysia) Malaysia) and were of analytical or HPLC grade. Pectinex Ultra SP–L was obtained from Science Technics (Science Technics SDN.BHD, Selangor, Malaysia). Standards for sucrose, glucose and fructose, as well as citric and ascorbic acids were obtained from Fluka Chemicals (New York, NY, USA). Linearity of the response to RI and UV detection was tested for each compound at five different concentrations prepared in distilled water and all resulting correlation coefficients were in the required range.

Betacyanin extraction

Extraction of pigments were achieved by homogenization of equal ratio of fruit pulp and solvents (1/1 w/v). Typically 100 g of the peeled fruit (pH=4.5; TSS=10%), of watery consistency, was shaken and macerated with 100 mL solvents (EtOH, aqueous ethanol 50:50) for 15 minutes under ice cooling condition. The aqueous mixture was centrifuged at 18,000 rpm and 4˚C for 20 min followed by fast filtration on nylon mesh. Yielded extract was concentrated in vacuum at 35^{o}C, using rotary evaporator, to 3–4 mL. The ethanol was completely removed after concentration process and samples were kept in a dark vessel.

Enzymatic treatment

Pectinex™ Ultra SP–L was chosen because it is a well–known commercial preparation that is used in the food processing industry for hydrolyzing pectin (Nikolic *et al.,* 2007) Different enzyme concentrations between 0.1–2% w/v were used for their applicability to degrade the mucilagenous material and releasing thousands small seeds for better betacyanin recovery from the pulp of *H. polyrhizus* fruit. For each sample, 100 g fruit pulp (pH adjusted to 4 by adding food grade citric

acid) was put into a glass beaker. Enzymatic treatment was carried out by adding Pectinex Ultra SP–L at dosages from 0.1, 0.3, 0.5, 1, and 2% w/v. The mixtures were incubated (G24 environmental incubator shaker, New Brunswick Scientific, USA) for 2 h at 40°C with continuous shaking (250 rpm). After incubation, samples were placed in a 90°C water bath (Precision 180 Series, Precision Scientific Inc., Chicago, USA) for 5 min for enzyme inactivation. Samples were immediately centrifuged for 20 min at 4°C and 18,000 rpm. The supernatants were carefully collected and stored in dark vessels. Samples were immediately analyzed for sugars, organic acids, and betacyanin retention. Again 100 gr of peeled fruit pressed and filtered. Purified juice adjusted to pH 4 by adding food grade citric acid and it was treated using the same set of procedures without using enzyme. Final sample was used as control of enzymatic treatment. Experiments were carried out in duplicate.

Determination of sugar contents

Concentrated color extracts were analyzed for their content of individual sugars (glucose, fructose, and sucrose). Samples were diluted 50–fold in deionized water, then filtered through a 0.45 µm filter (Millipore) and transferred into a vial. For sugar analysis, a method described by Kafkas *et al.* (2006) was slightly modified using an HPLC system (Jasco Co., Tokyo, Japan) equipped with refractive index (RI) detector (Jasco RI–1530), a pump (Jasco PU–1580), a mixer (Jasco LG–1580–04), and a degasser (Jasco DG–1580–054). HPLC was run by a model 2000 Jasco Borwin GPC (Tokyo, Japan) chromatography manager system. Separation of sugars was carried out using a Supelcosil™ LC–NH_2 HPLC column (250×4.6 mm i.d.) with a particle size of 5 µm (Supelco Bellafonte, PA, USA) at room temperature. The flow rate was 1 mL min^{-1}. The samples were eluted isocratically by using aqueous MeCn (20/80v/v) as solvent. The different sugars were identified by comparison of their retention times with those of pure standards. The concentrations of these compounds were calculated from calibration curves of the respective sugars.

Determination of organic acid contents

To determine the contents of citric acid a method described by Versari *et al*. (2008) was modified: The diluted samples were analyzed using HPLC (Shimadzu LC 6A) consisting of a pump and a controller coupled to a UV–spectrophotometric detector (Shimadzu SPC 6A). Separations were performed on an Aminex HPX–87H 300×7.8 mm i.d. column with particle size of 8 µm (Bio–Rad Laboratories, California, USA) operating at 36 °C with a flow rate of 0.6 mL min^{-1}. Elution was completed using an isocratic condition of the solvent (0.008 N H_2SO_4). Detection was carried out at 210 nm. The different organic acids were identified by comparing their retention times with those of pure standards. The concentrations of these compounds were calculated from calibration curves of the respective organic acids.

The ascorbic acid content was determined using a method previously reported (Kafkas *et al.,* 2006) in a modified context: The liquid chromatographic apparatus (Shimadzu LC 6A) consisted of a pump and a controller coupled to a UV–spectrophotometric detector (Shimadzu SPC 6A). Separations were performed on a Licro CART® 250×4.6 mm i.d. with particle size of 5 µm (Merck, Darmstadt, Germany) operating at 36°C with a flow rate of 0.6 mL min^{-1}. Detection was carried out at a wavelength of 254 nm. Elution was performed isocratically with MeCN/acetic acid/water (20/5/75, v/v/v). Each component was identified by comparing its retention time to an authentic standard and quantified by external standard method.

Betacyanin quantification

Betanin equivalents (Bc) were quantified using an UV– spectrophotometer (Hitachi High–Tech Co., Tokyo, Japan) by applying the equation of Cai and Corke (1999):

$$Bc\ (mg\ L^{-1}) = (A*F*MW*1000/\varepsilon.L) \quad (1)$$

where A is the absorption value at λ_{max} (540 nm), F is the dilution factor, MW is the molecular weight of betanin (550 g mol^{-1}), ε is the molar extinction coefficient of betanin (60,000 $mol^{-1}cm^{-1}$), and L (1 cm) is the pathlength of the cuvette. All determinations were carried out in triplicate.

Determination of betacyanin retention

HPLC analysis of concentrated betacyanin extract was carried out with a liquid chromatographic apparatus (Waters, Ca, USA) equipped with a pump Waters 600 controller, and a UV–Vis detector (Waters™ 486 Tunable Absorbance Detector). An analytical Lichrocart® Purospher ®Star RP18–column (250×4.6 mm i.d.), with a particle size of 5 µm (Merck, Darmstadt, Germany) was used for pigment analyses. The separation was performed isocratically using a mixture of 90 % solvent A (0.5 % aqueous TFA) with 10% solvent B (MeCN) for 40 min at a flow rate of 1 mL/min (injection volume: 10 µL). Detection was carried out at wavelength of 540 nm, relative chromatogram areas, as well as their specific ratios for the major betacyanins were calculated therefrom. All determinations were performed in duplicate. Individual betacyanin composition (%) was measured by analytical HPLC and was expressed as peak area percentages.

Quantitative and statistical analyses

Quantification of sugar, organic acids were based on external standard calibration method using linear regression analysis (r>0.999). For the stock solutions, the citric acid standards were dissolved in deionized water. Standard solutions for ascorbic acid required fresh preparation in 10% meta–phosphoric acid for stabilization purposes. Sugar references (glucose, fructose and sucrose) were dissolved in deionised water. All the samples and standards were injected three times each and mean values were used. The results were statistically evaluated by one–way analysis of variance (ANOVA). Statistical differences with p–values under 0.05 were considered significant and means were compared by Tukey's test, using MINITAB statistical software, V13.20 (MINITAB Inc., USA).

Results and discussion

Betacyanin extraction using enzymatic treatment

The high concentration of pectin present in the fruit (Esquivel *et al.,* 2007) will increase the viscosity of the final extract. Therefore, enzymatic treatment is required to hydrolyze the mucilage thereby allowing seed removal and thus improve production yield. The enzyme applied (Pectinex Ultra–SPL) isolated from *Aspergillus aculeatus* characterized by a high pectolytic activity thus appeared to be suitable. This enzyme is active in a temperature range of 15–55^{o}C and a pH range of 2.8–4.5 (Tran *et al.,* 2008). The activity threshold of this enzyme was considered suitable for the processing scheme selected. The duration and condition of the treatment were kept constant for all the samples and was a compromise between low thermal load to protect pigments and a temperature in the activity optimum of the enzyme. Different enzyme dosages were chosen to optimize product yield and degrade the pectin–like material. Visually, no color changes were observed in the final concentrated extracts. To get an accurate insight into betacyanin susceptibility to the enzyme preparation at different dosages, HPLC analyses was performed.

Sugar and acid constituent in concentrated betacyanin extracts

Table 1 shows the analytical results obtained from the concentrated betacyanin preparations. Organic acid analyses were carried out on concentrated extracts from solvent assays as well as the control sample. Ascorbic acid was present in very small amounts consistent with earlier findings (Esquivel *et al*., 2007) and thus did not contribute to acidity nor putative pigment stabilization.

Table 1. HPLC qualitative and quantitative data of sugars and organic acids in concentrated betacyanin preparations from *H. polyrhizus* fruit pulp, applying different extraction methods.

Simple	pH	Fructose[a] (g L^{-1})	Glucose[a] (g L^{-1})	Glucose/ fructose	Sucrose[a] (g L^{-1})	Citric acid[a] (mg L^{-1})	Ascorbic acid[a] (mg L^{-1})	Sugar–acid ratio[b]
Enzymatic treatment								
Tested enzyme concentrations (% w/v)								
0.1	4.03(±0.02)[a]	19.0(±0.1)	52.8(±1.1)	2.8	19.8(±0.7)	502.5(±3.5)[f]	0.6(±0.0)[b]	21:1
0.3	4.02(±0.01)[a]	20.2(±0.1)	52.6(±0.6)	2.5	20.1(±0.7)	678(±2.8)[e]	0.7(±0.0)[ab]	21:1
0.5	4.08(±0.01)[b]	20.5(±0.7)	51.1(±1.3)	2.5	20.9(±0.5)	650(±0.7)[d]	0.55(±0.07)[b]	15:1
0.75	4.12(±0.00)[b]	20.5(±2.1)	52.4(±0.2)	2.6	22.6(±0.9)	617.5(±3.5)[c]	0.7(±0.0)[ab]	25:1
1	4.03(±0.02)[a]	19.1(±0.1)	51.9(±2.1)	2.7	19.7(±1.7)	639(±1.4)[b]	0.7(±0.07)[ab]	22:1
2	4.02(±0.00)[a]	19.2(±0.3)	52.1(±1.5)	2.7	19.8(±1.1)	689(±1.4)[a]	0.9(±0.14)[a]	24:1
Solvent extraction								
EtOH	4.5(±0.07)[b]	42.5(±0.07)[b]	106.1(±14.21)[b]	2.5	35.9(±9.9)	476.5(±4.9)[c]	2.3(±0.21)[b]	56:1
EtOH:H_2O	4.6(±0.03)[b]	26.6(±5.37)[a]	56.5(±2.26)[a]	2.1	24.6(±1.5)	441.5(±4.9)[b]	1.0(±0.70)[ab]	38:1
Control[c]	4.2(±0.02)[a]	19.6(±0.21)[a]	50.1(±0.05)[a]	2.5	19.5(±0.7)	444(±5.6)[a]	0.5(±0.00)[a]	16:1

[a–f] Means in the same column are significantly different at $p < 0.05$ using Tukey's HSD test.
[a] Mean values of duplicate measurements ± standard deviation (±SD).
[b] Calculated as total sugars/total acids.
[c] purified juice.

Analysis of the sugar composition revealed that *H. polyrhizus* contained glucose, fructose and sucrose. The presence of sucrose in *H. polyrhizus* fruits was detected in all experiments, although Stintzing *et al.* (2003) reported these fruits were devoid of sucrose. It is suspected that these contradicting results are due to different methods used. While Stintzing *et al.* (2003) had assessed sugars by enzyme test kits, the present study used HPLC as a more reliable and accurate assay. In addition, the invertase activity of the fruits may be diverse thus explaining the diverting results (Esquivel *et al.*, 2007).

Tukey's means difference test for extracts produced by enzymatic treatments showed no significant ($P>0.05$) differences in sugar contents. Mean values for fructose and glucose were found statistically significant ($P<0.05$) in solvent–extracted samples. Considerable amounts of sugars were released from cell walls during ethanolic extraction. In the enzyme–treated samples with dosages of 0.3 % w/v and 0.5 % w/v the glucose/fructose ratios of 2.5 were found to be higher with the 0.5% level. In general, higher content of sugars and acids result compared to untreated sample (control) through enzyme treatment. From an industrial point of view, the sugar–acid ratio should be at about 10–18:1 to match the required sensorial quality of the final product. Typically, Hylocereus juices range between 11:1 to 35:1 (Stintzing *et al*., 2003; Esquivel *et al.,* 2007). By acidification and enzymation ratios of 15:1 to 22:1 resulted when the lower dosage of enzyme was applied. On the other hand, higher levels of organic acids were considered to be of interest due to their impact on color and pigment stability (Herbach *et al.,* 2006a), a welcome side–effect.

Individual betacyanin patterns in concentrated extracts

Betacyanin retention in each concentrated extract was monitored by HPLC. The different stabilities of the betacyanins for each sample were assessed by comparing peak areas of the respective pigments treated by different enzyme dosages (Table 2). Figure 1 (A–F) shows chromatograms of betacyanins in H. polyrhizus extracts prepared by enzymatic treatments. Betanin, phyllocactin, hylocerenin, and their respective isomers were identified as the major betacyanin components in the pulp of *Hylocereus polyrhizus* fruits extracted by enzymes. The content of betanin, isobetanin, phyllocactin, hylocerenin as well as isophyllocactin and isohylocerenin was measured. By peak area comparison of untreated sample (control) and enzyme–treated samples betanin and isobetanin content were found to decline to 20% of the initial value with increasing enzyme dosage, while phyllocactin declined by 39%. In contrast, isophyllocactin was found to amount to 58% of the peak area ratio at the highest enzyme dosage (2% w/v). In accordance with previous findings (Herbach *et al.,* 2007) betanin and isobetanin pigments showed higher stability over the entire treatment range in this study. In the sample treated with the lowest dosage of enzyme, isohylocerenin was not detected, whereas after treatments with higher dosage of enzyme isohylocerenin concentration gradually increased to 7% (Figure 1).

In all enzyme–treated samples phyllocactin was the least stable and degraded faster compared to the other betacyanin pigments. This is in analogy to the thermal stability of betacyanin pigments investigated in several studies (Herbach *et al.,* 2004, 2006a) when phyllocactin displayed lowest stability among pitaya pigments. Considering that pH, temperature, and time were kept constant in our study, the correlation between the enzyme dosage and relative concentration area of the predominant betacyanin fractions should indicate the individual pigment liability to structural alteration As demonstrated in Figure 2, almost 99% of the concentration variation of betanin, phyllocactin, and isophyllocactin was ascribed to the enzyme dosage.

Table 2. Relative chromatogram areas and betacyanin ratios of major betacyanins in concentrated color extracts from *H. polyrhizus* fruits.

Betacyanin	R_t	Enzyme concentration (w/v)							EtOH	EtOH:H_2O
	min	Control	0.10 (%)	0.30 (%)	0.50 (%)	0.75 (%)	1 (%)	2 (%)		
Betanin	7.8	31.67	30.66	27.85	25.19	25.01	22.11	12.95	30.34	30.78
Isobetanin	9.5	5.46	5.17	4.93	4.74	4.99	4.61	3.19	4.43	4.69
Phyllocactin	18.3	50.70	46.78	38.44	33.53	30.99	22.79	11.37	52.14	52.26
Hylocerenin	22.2	11.14	11.43	10.63	10.54	10.03	8.33	6.14	11.09	11.07
Isophyllocactin	23.8	1.02	5.97	16.50	23.72	28.91	37.79	59.24	2.01	1.19
Isohylocerenin	33.7	–	–	1.65	2.27	2.19	4.38	7.11	0.00	0.00
Betanin:phyllocactin	–	0.62	0.66	0.72	0.75	0.81	0.97	1.14	0.58	0.58
Betanin:hylocerenin	–	2.80	2.70	2.60	2.40	2.50	2.70	2.10	2.70	2.70
Phyllocactin:hylocerenin	–	4.50	4.10	3.60	3.20	3.10	2.70	1.80	4.70	4.70

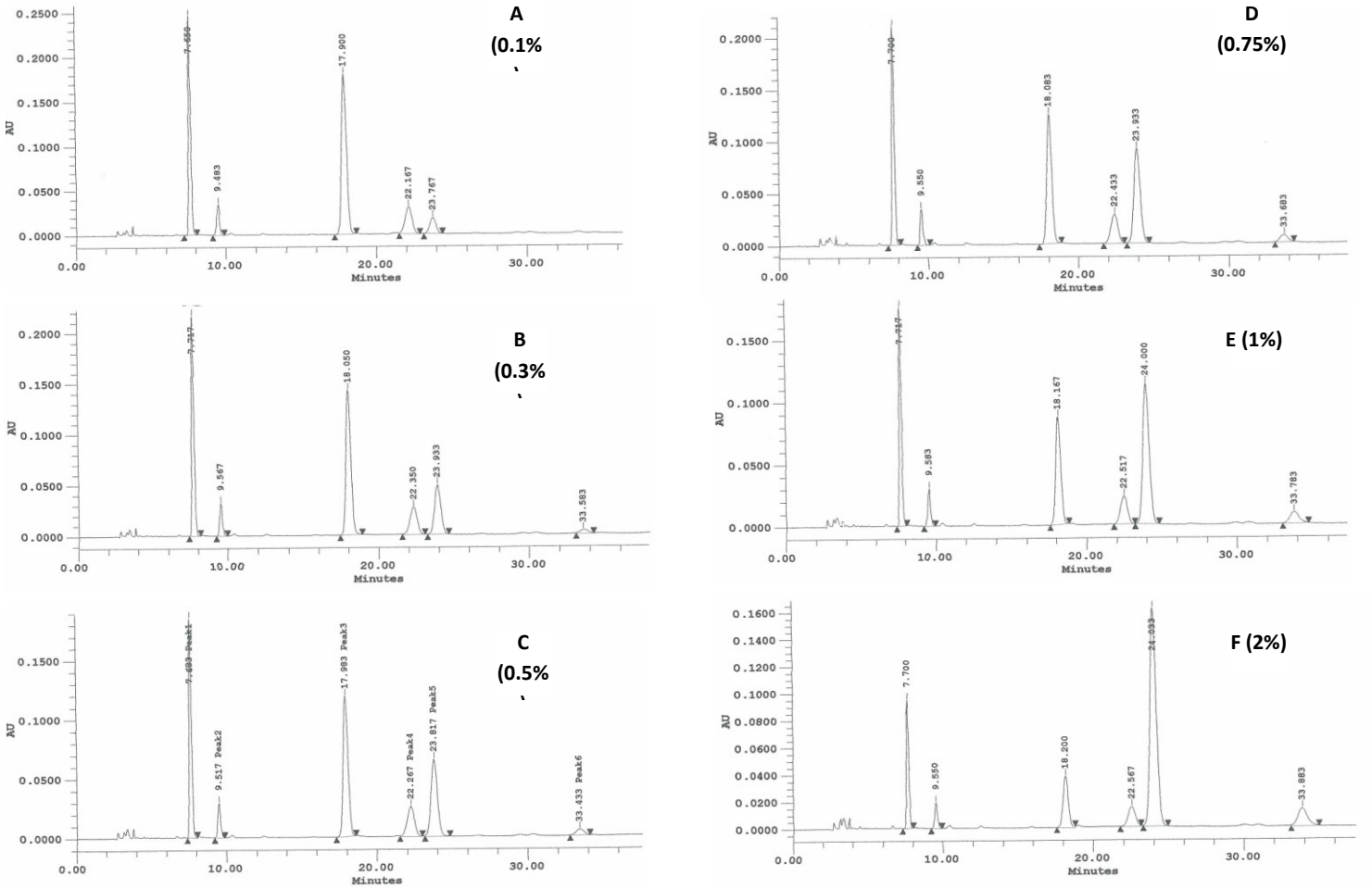

Figure 1. HPLC profile of concentrated betacyanin extract obtained through enzymatic treatment with different dosage of enzyme (0.1–2% w/v; from A–F).

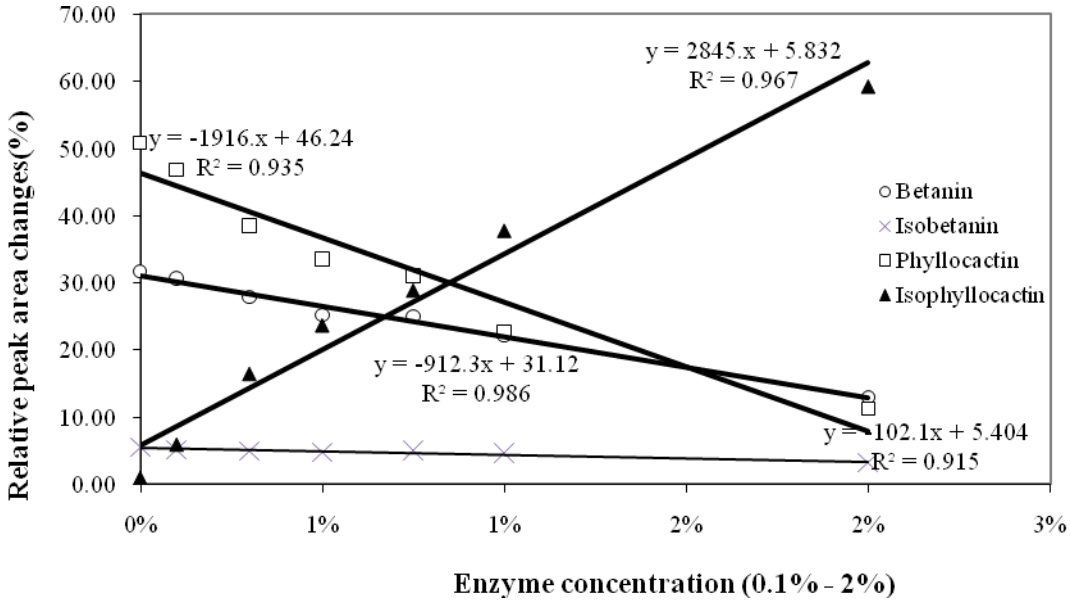

Figure 2. Relationships between enzyme concentration and predominant betacyanin pigments.

Betacyanin content, retention and conversion

Betacyanin concentration of *Hylocereus* preparations is economically important with respect to its possible use as a colorant. Betacyanin content in concentrated extracts was monitored by spectrophotometry (Table 3). For the determination of betacyanin stability and the effect of enzyme dosage, both overall betacyanin retention and the impact on the major betacyanins were evaluated.

Betacyanin retention in enzymatic experiments calculated as betanin was highest in the sample treated with 0.3% (w/v) enzyme. Individual stabilities of the different betacyanins in enzyme–treated extracts were monitored by comparing their peak area ratios (Table 2). The isomerization index expressed as betanin/isobetanin, and the deacylation index, expressed as betanin/phyllocactin, and the phyllocactin and hylocerenin isomerization index were evaluated in detail by comparing their peak area ratios (Table 3). The isomerization index was not altered in the samples treated with the lower enzyme dosage (0.1%–0.5% w/v). It was observed that treatments with higher concentration of enzyme induced further decline of the isomerization indices. The peak area ratio betanin/phyllocactin of 0.6 in samples treated by the lower of enzyme dosages (0.1%, 0.3% w/v) increased. This can be ascribed to deacylation of phyllocactin resulting in betanin formation (Herbach *et al.,* 2006ab). Since this ratio levelled off to 1.0 for the higher enzyme–treated concentrations (1%, 2% w/v), a negative effect of enzyme dosage on betacyanin pigments can be easily deduced from calculating the peak area ratio of betanin and phyllocactin. By comparing total peak area and individual major betacyanins in the enzyme–treated concentrates, betanin and isobetanin presented highest stability in all treatments, while phyllocactin degraded faster (Figure 3) as further reflected in a drastic decline of the phyllocactin/isophyllocactin ratio (Table 3). It is woth noting that no aglyca were detected which are known to be highly labile and are an indication for a strong beta–glucosidase side activity of the enzyme applied.

Betacyanin fingerprints in several studies showed differences not only in different species but in the proportion of certain pigments in the same species (Esquivel *et al*., 2006; Wybraniec and Platzner, 2002). On the other hand, it has not yet been clarified whether co–occurring betacyanin C15–isomers are mainly isolation artifacts (Stintzing and Carle, 2007). Additionally, Herbach *et al*.

(2006c) observed that the higher stability of betacyanins in *Hylocereus polyrhizus* compared to betacyanins in red beet was not due to the genuine acylated phyllocactin and hylocerenin pigments in *H. polyrhizus*, but rather due to the higher stability of the heat–induced artifacts. Interestingly, C15–isomerization of betacyanins was induced by enzymatic treatment in the present study. This result may be explained by the fact that a selective adsorption process to pectin fragments of different sizes in the matrix will alter the mobility of the pigments and thus their mutual interactions and alteration during the treatments (Stintzing and Carle 2007).

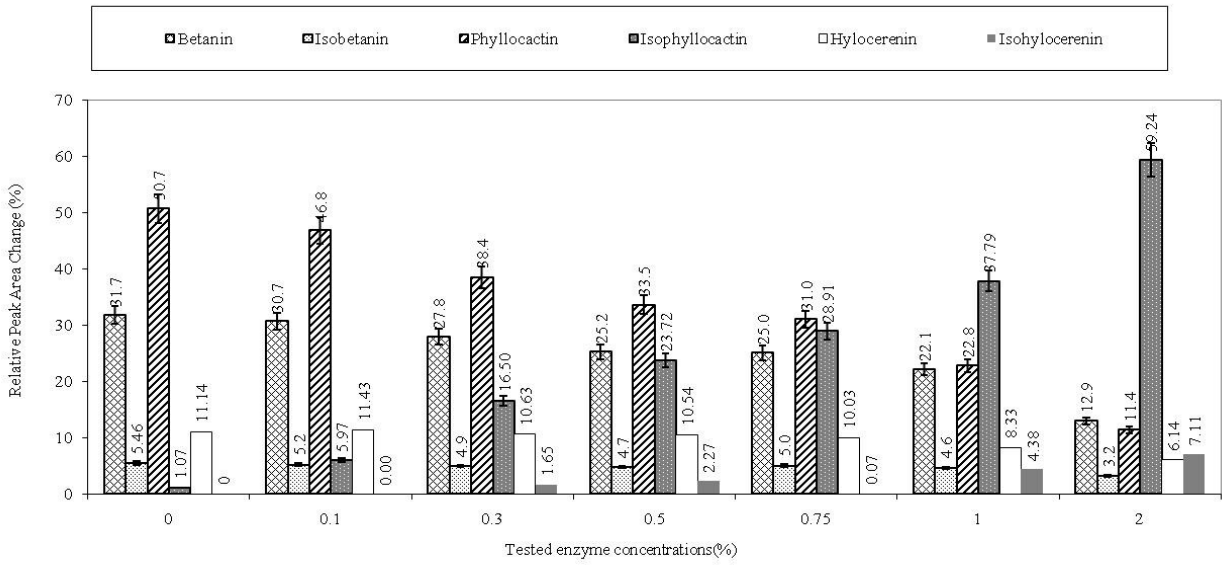

Figure 3. Relative peak area changes (%) of betanin, isobetanin, phyllocactin, isophyllocactin, hylocerenin and isohylocerenin peak areas in extracted colorants.

Table 3. Betacyanin retention in extracted colors obtained through enzymatic treatments monitored at λ_{max} by spectrophotometric and HPLC analyses.

Sample[a]	Spectrophotometer (mgL^{-1})	Isomerization ratios in enzyme–treated samples (HPLC analysis[b])			
		Betanin/isobetanin	Betanin/ phyllocactin	Phyllocactin/ isophyllocactin	Hylocerenin/ isophylocerenin
Tested enzyme concentrations (% w/v):					
Control	498.6 (±5.18)	6.0	0.6	49.5	–
0.1	490.4 (±6.48)	6.0	0.6	7.8	–
0.3	550.0 (±0.00)	6.0	0.6	2.0	6.4
0.5	302.5 (±0.00)	6.0	0.7	1.4	4.6
0.75	366.7 (±0.00)	5.0	0.75	1.1	4.5
1	288.8 (±19.45)	5.0	1.0	1.0	2.0
2	275.0 (±0.00)	4.0	1.0	0.2	0.8

Values given are means of triplicate determinations ± standard deviation.
[a] extracted colorant by enzymatic treatment.
[b] expressed as relative chromatogram areas.

Conclusions

The analytical control of betacyanin compositions in extracted colorants showed some difference in their stractures in each extraction mode. Betanin showed the most stable structure compare to phyllocactin and hylocerenin. In enzyme treated samples increasing dosage of enzyme was reflected in phyllocactin and hylocerenin isomerisation which was monitored by HPLC analysis. Best pigment release was obtained at 0.3% enzyme dosage. In particular, while enzymatic treatment can be scaled up to extract color from the pulp of *Hylocereus polyrhizus* fruits as well as easier seed removing task, further studies are under way to improve pigment retention and it will be the subject of future studies.

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