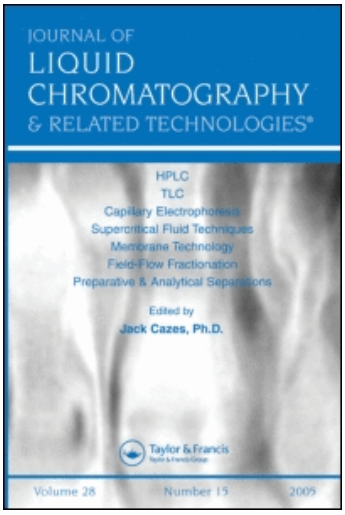

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Analysis of Polyphenols from Hop Bract Region Using CCC

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Abstract: Polyphenols derived from hop (*Humulus lupulus* L.) bract region (HBP) can be used as food materials, thereby preventing dental caries. Chemical details of the active substances need to be elucidated. The polyphenols from hop bract (HBP) region were purified by countercurrent chromatography (CCC). The fractions were analyzed by high-performance size-exclusion chromatography (HPSEC) and reversed phase high-performance liquid chromatography (RP-HPLC). From HBP fractions by HPSEC, some low-molecular- weight polyphenols (glycosides of flavonoids, catechins, and proanthocyanidins) were identified by RP-HPLC. However, a very hydrophilic fraction was found to have the most potent cavity-preventive activity, but it showed no peak in its RP-HPLC chromatogram (absence of small polyphenols). HPSEC analysis showed that the major components of this fraction were

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high-molecular weight substances, which were supposed to be proanthocyanidins, consisting of approximately 22 catechin units in its structure.

Keywords: Countercurrent chromatography, Hop (*Humulus lupulus* L.), Proanthocyanidin, Glucosyltransferase

INTRODUCTION

Hop is not only used for beer production, but is also known as an herbal plant, which has several positive functions for human health. For example, hop has been reported to have antioxidative[1] and female hormone-like activity.[2] The lupline gland region of hop cone is utilized for beer production, but most of the other bract region is used only as cattle feed. We have reported that polyphenols derived from hop bract region have potent cavity-preventive activity, but the chemical structure of active compound has not been clarified in detail.[3] As hop-derived polyphenols, catechins (catechin and epicatechin), phenylpropanoids (xanthohumol, 8-prenyl naringenin, etc.), and glycosides of fravonoid (rutin, isoquercitrin, etc.) are well known.[2–4] The presence of 2–20 mer proanthocyanidins, analyzed by TOF-MS spectrometry, has also been reported.[4] Proanthocyanidin is the generic name for oligomers and polymers consisting of catechins as a constituent unit, and it is present in various foods such as apples, grapes, and the skin of beans. Its functions, such as antioxidative,[5–7] antitumor,[8] antibacterial,[9] and antihypertensive actions,[10] and prevention of arteriosclerosis[11] have been reported. However, purification and detection of the high molecular weight proanthocyanidin is difficult because of the presence of many homologues and isomers, resulting in delay of proceedings of its analytical investigations.[12] To elucidate the mechanism of its positive effects on human health, establishment of an analysis method for proanthocyanidin is strongly desired.

Countercurrent chromatography (CCC) is an analytical method for separation of various complex substances using a two-phase solvent system. Since CCC uses two mutually immiscible liquid phases, it has two advantages for performing both ‘liquid-liquid extraction’ and ‘chromatography.’ Its application has recently been expanded to separation of natural or synthetic substances, as well as samples which are difficult to purify by high performance liquid chromatography (HPLC). For example, easily-degraded substances and substances that irreversibly adsorb onto the solid support in column chromatography can be handled by CCC. Since CCC uses a liquid stationary phase, no degradation, irreversible adsorption, or loss of solutes occurs during passage through the column. According to the recent report, procyanidin oligomers contained in apple condensed-tannins (ACT) have been efficiently separated by CCC.[13–15]

In this study, we separated and fractionated polyphenols containing high molecular weight proanthocyanidins from the hop bract region (HBP) by

CCC. It was shown that the cavity-preventive activity increased with the increase in the polarity of HBP fractions by high performance size exclusion chromatography (HPSEC). The active fractions were analyzed by UV measurement, RP-HPLC, and HPSEC.

EXPERIMENTAL

Extraction of Polyphenols from Hop Bracts

Polyphenols were prepared as previously described.[3] Briefly, 10 kg of hop bract region were added to 150 L of 40% ethanol, stirred at 30°C for 1 hour, and filtered to clarify the solution. The clarified hop solution was passed into an 8 L SP850 column (Mitsubishi Chemical, Tokyo, Japan), and the polyphenol fraction was eluted with 50% aqueous ethanol mobile phase. The eluate was then concentrated and spray-dried, yielding 190 g of polyphenol fraction (HBP) at 1.9% (w/w) (3.6 g of pure polyphenols). To defat the obtained polyphenol fraction, 100 g were dissolved in 200 mL of pure water, and extracted 3 times with 200 mL of hexane. The aqueous phase was then extracted 3 times with 100 mL of dichloromethane and centrifuged at 3,500 rpm for 10 min. After centrifugation, the aqueous solution was concentrated using a rotary evaporator, and lyophilized to give 88 g of brown amorphous powder.

Reagents

Organic solvents, including acetonitrile (CH_3CN), methyl t–butyl ether (MTBE), and trifluoroacetic acid (TFA) were all of analytical grade (Kanto Chemicals, Tokyo, Japan). Polyphenolic standards, including catechin, rutin, isoquercitrin, kaempferol-rutinoside, and kaempferol-glucoside were purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Proanthocyanidins (2–5 mers) were purified from ACT by normal-phase HPLC.[16] Other chemicals were all of reagent grade.

HBP Fractionation by CCC

CCC fractionation was performed using a type-J coil-planet centrifuge (J-CPC) manufactured by Renesas Eastern Japan Semiconductor Inc., Tokyo, Japan.[13] The J-CPC was connected to the Hitachi (Tokyo, Japan) HPLC instruments with an L-7100 pump, a Reodyne 7166 sample injector, and an L-7455 diode-array detector. The separation column in the J-CPC was fabricated by winding a single piece of PTFE tubing (1.0 mm I.D. × 2.0 mm

O.D. × 50 m length) directly onto the holder hub, making six coiled layers between a pair of flanges ($\beta = 0.5–0.6$). The total capacity of the column was about 40 mL. The solvents of the two-phase system were MTBE:CH_3CN:0.1% aqueous TFA (2:2:3 v/v/v). The upper organic phase of the solvent system was used as mobile phase and the lower aqueous phase was the stationary phase (normal phase mode).

In each CCC separation, the coiled PTFE column was first entirely filled with the lower aqueous and polar stationary phase and, next, the column was rotated at 1,250 rpm while the upper organic mobile phase was pumped into the column in the tail to head (T → H) direction at a flow-rate of 2.0 mL/min. After the hydrodynamic mixing between the two phases reached a state of equilibrium in the column, the stationary phase retention ratio was estimated to be $S_f = 73\%$ ($V_S = 29$ mL). One mL of a sample solution containing 25 mg of HBP dissolved in the aqueous phase was injected. The UV absorbance of the eluate (upper organic phase) was continuously monitored, and collected in 2.0 mL (1 min) tubes using an SF-2120 fraction collector (Advantec, Tokyo, Japan). About 60 minutes (120 mL) later, the CCC run was stopped, and the column was continuously re-rotated at 1,250 rpm while the lower stationary phase was pumped into the column in a head to tail (H → T) direction the same flow-rate of 2.0 mL/min (dual-mode method). The UV absorbance of the eluate (lower phase) was monitored, and similarly collected in 2 mL tubes. The contents of the tubes were concentrated using a rotary evaporator, and lyophilized to give amorphous powders.

RP-HPLC Analysis

The fractions obtained by CCC were analyzed by RP-HPLC under the following conditions: column: Mightysil RP-18GP (4.6 mm I.D. × 150 mm, 5 μm) (Kanto Chemicals); mobile phase: A = 0.1% TFA: CH_3CN (90:10 v/v), B = 0.1% TFA: CH_3CN (50:50 v/v). Gradient, 0 → 20 min, 100% A; 20 → 60 min, 50% A (50% B); injection volume: 10 μL; flow rate: 1 mL/min; detection: 280 nm; column temperature: 25°C.

HPSEC Analysis

The fractions obtained by CCC were analyzed by HPSEC, according to the Taylor's method,[4] under the following conditions: instrument: Agilent 1100 series Agilent ChemStation; columns: two Agilent Technologies PLgel columns, 500-A and 100-A (7.6 mm I.D. × 300 mm); mobile phase: N,N-dimethylformamide (containing 10 mM LiBr); flow rate: 0.5 mL/min; column temperature: 40°C; detection: UV 280 nm.

UV Profile Measurement

Samples were dissolved in 20% methanol in water at a concentration of 100 μg/mL and the absorbance was measured at 220–400 nm using a UV-2000 spectrophotometer (Hitachi, Ltd., Tokyo, Japan).

Enzymatic Assay for Dental Plaque-Forming Enzyme

The inhibitory activity against glucosyltransferase (GTF), which is the dental caries-causing enzyme, or the dental plaque-forming enzyme, was evaluated.[3,17] Briefly, 1,000 μL of substrate solution (100 mM phosphate buffer, pH 6.5, containing 2% sucrose, 0.1% NaN_3, 40 mM dextran T10), 150 μL of sample solution, 10 μL of GTF enzyme solution (from Streptococcus sobrinus 6715), and 340 μL of distilled water were mixed. This mixture was incubated at 37°C for 18 hours, and the turbidity was measured at 550 nm using an ultraviolet-visible spectrophotometer (UV-2000, Hitachi, Ltd). Designating the control as 100%, the insoluble glucan formation rate was calculated by the equation below (OD = optical density):

$$\begin{aligned}&\text{Insoluble glucan formation rate (\%)}\\&= [(OD_{\text{sample}} - OD_{\text{enzyme blank of sample}})/\\&\quad (OD_{\text{control}} - OD_{\text{enzyme blank of control}})] \times 100 \qquad (1)\end{aligned}$$

RESULTS AND DISCUSSION

Fractionation of HBP by CCC

The prepared hop bract polyphenol (HBP) sample was fractionated by CCC. A total of 100 mg of HBP (25 mg × 4 times) was separated and divided into 10 fractions by pooling together the collected tube corresponding to the peaks seen in the CCC chromatogram (Fig. 1). Fractions 1–7 were obtained by eluting the organic upper phase (aqueous phase stationary, normal phase mode). Fractions 8–10 were obtained in reversed phase mode by eluting the aqueous lower phase after reversing the flow direction (dual-mode method). Since the upper organic phase is the least polar phase, when it was used as the mobile phase, the hydrophobic components should be eluted first followed by elution of components with an increasing order of polarity. The dry weights of the fractions are shown in Table 1. The total yield was about 93%, showing that almost the entire amount of fractionated sample was recovered. The most hydrophilic fraction, Fraction 10 showed the highest absorbance at UV 280 nm and had the heaviest dry weight, accounting for about 40% of the total dry weight. Based on the reported analysis of ACT,[13–15] Fr. 10 may contain a mixture of highly polymerized

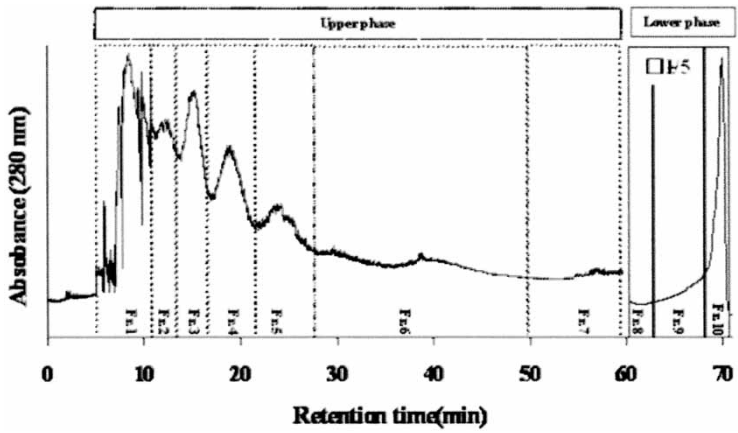

Figure 1. Countercurrent chromatographic separation of HBP. Eluent was separated into 10 fractions according to lines shown in figure. Absorbance of fractions 8–10 was shown in 1/5 scale of fraction 1–7.

proanthocyanidins with more than 10 mer. This result is consistent with the previous report that a high-molecular-weight fraction separated by ultrafiltration accounted for about 50% of dry weight of HBP.[3]

RP-HPLC Analysis of HBP Fractions by CCC

The 10 fractions separated by CCC were analyzed by RP-HPLC to confirm their compositions (Fig. 2). It was consistent with the previous report that

Table 1. Dry weights of HBP fractions obtained from CCC

Fraction number	Content (mg)
1	4.4
2	5.3
3	5.1
4	6.5
5	6.7
6	6.7
7	4.2
8	4.7
9	7.8
10	41.3
Total	92.7

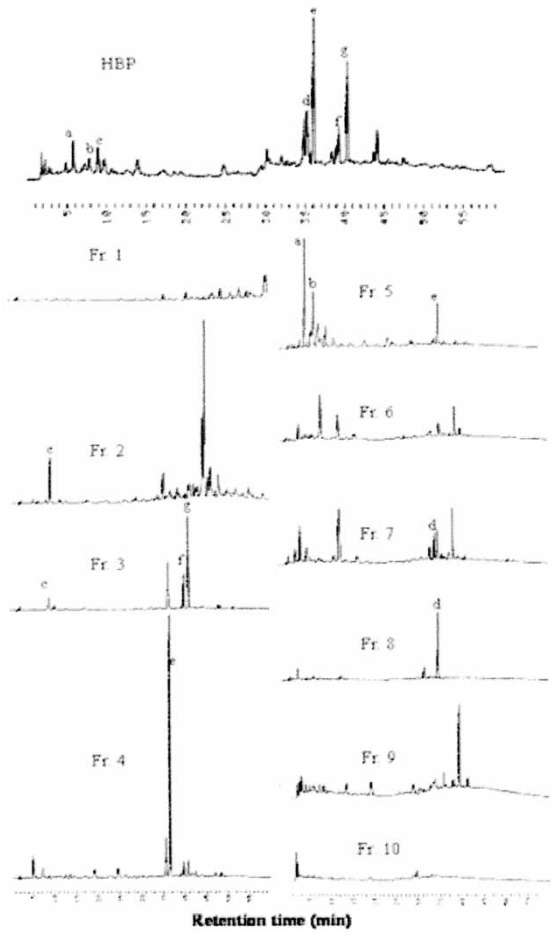

Figure 2. Reversed-phase HPLC chromatogram of HBP fractions. a, procyanidin B1; b, procyanidin B3; c, catechin; d, rutin; e, isoquercitrin; f, kaempferol-3-rutinoside; g, kaempferol-3-glucoside. Quantities of injected sample: 20 μg (fraction 1, 2, 5–7, 9, 10); 5 μg (fraction 3, 4, 8). Detection: UV 280 nm.

catechin was detected in Fractions 2 and 3 eluted earlier than procyanidin B1 (PB1) and B3 (PB3), which were the dimer of catechins (Fr. 5). Comparing analysis of ACT[13–15], which mostly consisted of proanthocyanidin, it was shown that HBP fractions contained some glycosides of flavonoid, such as kaempferol-3-glucoside (Fr. 3), kaempferol-3-rutinose (Fr. 3), isoquercitrin (Fr. 4), and rutin (Frs. 7 and 8). Although Fr. 10 showed a high absorbance in the CCC chromatogram and represented a large amount of dry weight, it gave no clear peak in the RP-HPLC chromatogram detected at 280 nm. Since Fraction 10 was the most hydrophilic fraction, the substances present

in the fraction should not be irreversibly adsorbed onto the solid ODS stationary phase. They may be highly retained due to their molecular sizes. At a shorter wavelength, such as UV 220 nm, a very broad elution peak, i.e., a 'broad mound,' was observed in the RP-HPLC chromatogram (data not shown). This result is consistent with the finding that no clear peak was detected by RP-HPLC in the high molecular weight polyphenols obtained by ultrafiltration.[3] Thus, it was supposed that the major components of Fr. 10 were highly polymerized polyphenols, consisting of various homologues and isomers, hence, resulting in failure of the ysis by RP-HPLC. Generally, RP-HPLC is widely used in analytical studies of natural products and, in practice, only a substance which gives a sharp peak in the HPLC chromatogram, is dealt with. However, the HPLC results obtained with Fr. 10 of HBP suggest that analysis only by RP-HPLC may not be sufficient. A combined study with RP-HPLC and CCC analyses would improve this situation: the Fr. 10 content could be further fractionated by CCC using a more polar biphasic liquid system.

HPSEC Analysis of HBP Fractions by CCC

To elucidate the molecular weight profile of components, the CCC fractions obtained from HBP were analyzed by HPSEC. As shown in Fig. 3, it revealed that the molecular weights of HBP fractions tend to increase with the fraction number, which is consistent with the previous report that CCC separated procyanidin oligomers by the degree of polymerization.[13,14] The peak tops of Frs. 1–4 were observed at about 31 min and they shifted leftward as the fraction number increased, indicating that a relative amount of high molecular weight substances increased with retention time. For example, the peak top of Fr. 10 was eluted at about 23 min with a minimum amount of low molecular weight substances at 31 min. Using purified procyanidin oligomers (1–5 mer) from ACT as the standard sample,[16] the molecular weight of these peaks was evaluated (Fig. 4A). Their log M.W. versus retention time curve was reasonably linear (Fig. 4B). Using this standard curve, the peak at 31 min in Fig. 3 was evaluated to be a monomer of catechin (M.W. = 290). If the valid time range of the standard curve can be expanded, the molecular weight of the main peak of Fraction 10 (peak h in Fig. 3) is evaluated to be a 22 mer of procyanidin (M.W. of about 6,300). Peak i in Fig. 3 (one of major peaks in HBP) was also evaluated as about 9 mer (M.W. of about 2,600). Although these evaluations are just approximate figures, they are consistent with a previous report mentioning that the hop contained 2–20 mer of proanthocyanidin.[4]

UV-Profile Analysis of HBP Fraction by CCC

To assess the chemical characteristic of the HBP fraction by CCC, the UV-profile of each fraction was taken between 220 and 400 nm. Figure 5

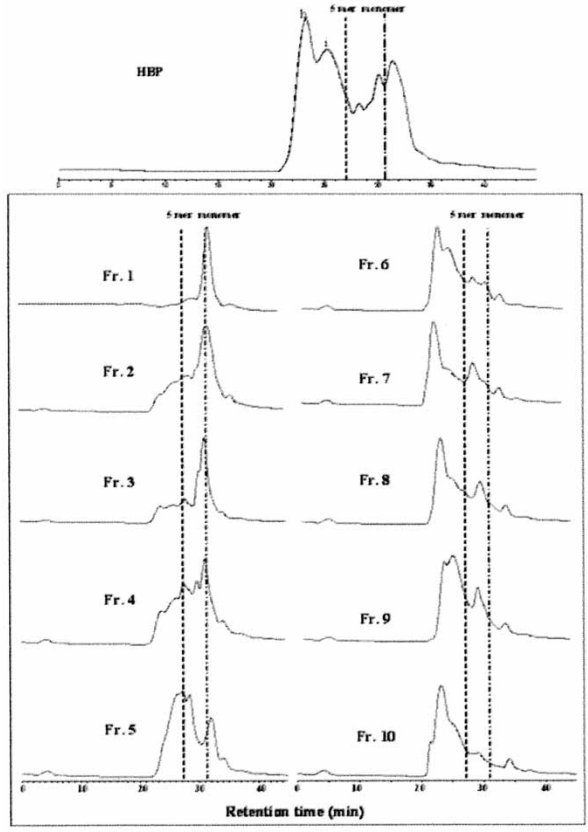

Figure 3. Size-exclusion chromatograms of HBP fractions.

shows UV-profile data of reagents (catechin and rutin) and representative HBP fractions (each 100 μg/mL). The UV-profile of catechin showed a peak maximum at 278 nm and a valley at 250 nm. Since the UV-profile of Procyanidin oligomers showed a good agreement with that of catechin,[16] proanthocyanidin seems to have a catechin-like UV-profile. Rutin shows two peaks at 256 and 352 nm that is a characteristic profile of a flavone structure such as quercetin and kampferol.

The UV-profile of HBP ($\lambda_{max} = 273$ nm) is somewhat similar to that of catechin, but with a much shallower valley at 250–270 nm and a trailing decrease in the 280–400 nm region. This result suggests that the major components of HBP were proanthocyanidins, i.e., polymerized catechins. Indeed, HBP develops a red color by acidic hydrolysis,[18] due to formation of cyanidin (data not shown). The difference in the UV-profile of HBP from that of catechin may be due to the presence of a flavone moiety in the molecule, or browning of proanthocyanidins during the purification process of HBP.

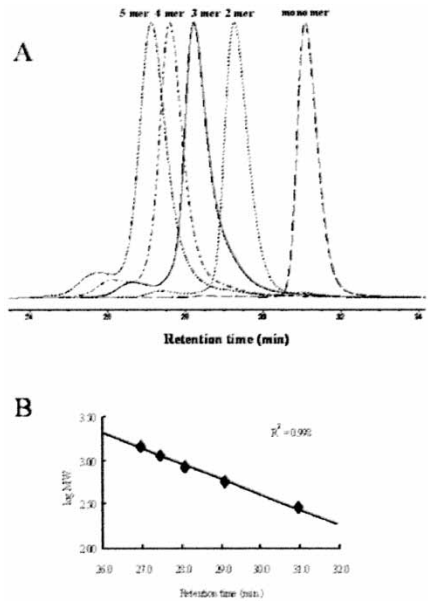

Figure 4. A: Size-exclusion chromatogram of procyanidin oligomers separated by normal-phase HPLC.[16] The vertical axis shows absorbance at 280 nm. B: Standard curve calculated from the data of Fig.4A.

HBP fractions were roughly classified into two groups according to their characteristic UV-profiles: one group (Fr. 2, 3, 4, and 8) showed a high absorbance peak at about 270 nm, and a smooth shoulder at around 320–370 nm. RP-HPLC analysis proved that these fractions similarly contained glycosides

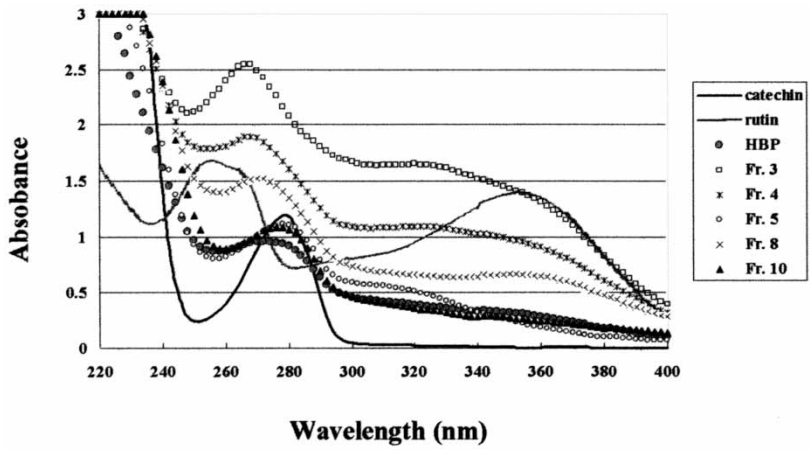

Figure 5. UV spectra of HBP fractions.

of flavone, thus reflecting the combined UV spectra of catechin and flavone structures. The other group (Fr. 5, 6, 7, 9, and 10) showed the UV-profile like HBP. Since Fr. 5 was found to contain procyanidin B1 and B3 as major components by RP-HPLC analysis, the major component of the other Fr. 6, 7, 9, 10 was also assumed to be the polymerized catechins.

Evaluation of Inhibitory Activity on Dental Plaque-Forming Enzyme

Dental plaque formation is considered to be an important factor of dental caries. Dental plaque is caused by glucosyltransferase (GTF), an enzyme produced by cariogenic bacteria (Mutans Streptococci.). Dietary sugars are synthesized into viscous insoluble glucan by GTF, through which bacteria adhere to the tooth surface and form dental plaques, causing cavity. Therefore, substances that inhibit GTF are expected to be effective cavity-preventive agents. Since previous studies have shown that polyphenols from hop bract region exhibit potent inhibitory effect on GTF,[3] the inhibitory effect of the HBP fractions obtained by CCC on the dental plaque-forming enzyme is most likely attributed to the polyphenols.

The results of inhibition of GTF activity (IC_{50} values) are shown in Fig. 6. The fractions 1–4, which were relatively hydrophobic, showed very weak inhibition on GTF, i.e., their IC_{50} values were over 100 $\mu g/mL$. As the hydrophobicity of the fraction decreased, the inhibitory activities on GTF increased. The IC_{50} values of Fractions 5–9 were, however, about 38–70 $\mu g/mL$, which were almost equivalent to or lower than that of HBP. Only Fraction 10, which is the most hydrophilic fraction and supposed to contain highly-condensed catechin polymers, strongly inhibited GTF with an IC_{50} value of 24 $\mu g/mL$, which was a lower concentration than that of HBP. This result suggests that

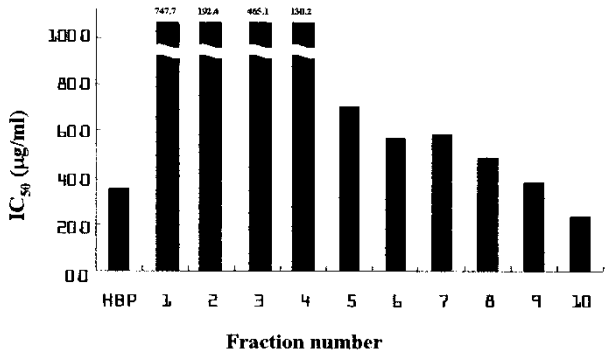

Figure 6. Fifty percent-inhibitory concentration of HBP fractions on dental plaque-forming enzyme.

high molecular weight (hydrophilic) polyphenols more strongly inhibit insoluble glucan production than low molecular weight (hydrophobic) polyphenols, as reported earlier.[3]

CONCLUSIONS

Using CCC, we analyzed HBP, of which the chemical composition has not been fully clarified. CCC efficiently fractionated HBP based on the differences in polarity, indicating that the most hydrophilic (highest in molecular weight) fraction strongly inhibited GTF, exhibiting the cavity-preventive activity. These active substances were assumed to be proanthocyanidins (polymerized catechins) on the basis of their UV-profile. The degree of polymerization was evaluated as ca. 22 by HPSEC. Further analytical studies of this fraction, especially focusing on the linking pattern between catechin units, are currently under way in our laboratory.

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