SELEKSI DARI KALUS YANG MENGHASILKAN ANTOSIANIN TINGGI PADA *POLYGONUM TINCHTORIUM* AIT

SELECTION OF HIGH MELDING ANTHOCYANIN CALLUS LINE OF POLYGONUM TINCHTORIUM AIT

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Abstract

*The effects of kind and concentration of plant growth regulators on anthocyanin synthesis of six cultivars of **Polygonium tinchtorium** Ait were studied. The cultivar tested were (1) Shoujyouko Shirobana, (2) Matsuae Ai, (3) Hyakkan, (4) Shouyouko Akabana, (5) Miyagi Ai and (6) Akaguki Kosenbon. Among those cultivars, cultivar no. 1, 2 and 3 did not synthesize anthocyanin, cultivar no. 4 produced slightly anthocyanin while cultivar no. 5 and 6 produced large amount of anthocyanin. The best condition to induce anthocyanin formation was kinetin (KIN)-indole acetic acid (IAA) (KIN 10 ppm; IAA 0, 5, 1, 3, 5, 10 ppm) under light condition using petiole as an explant. The amount of anthocyanin produced by selected callus was four times higher than unscreened callus. Indican was a little detected on MeOH extract from green callus.*

Ringkasan

Pengaruh dari jenis dan konsentrasi zat pengatur tumbuh pada sintesa antosianin dari 6 kultivar P. *tinchtorium* Ait telah dipelajari. Kultivar yang diuji adalah (1) Shoujyouko Shirobana, (2) Matsuae Ai, (3) Hyakkan, (4) Shoujouko Akabana, (5) Miyagi Ai, dan (6) Akaguki kosenbon. Diantara kultivar tersebut, kultivar no. 1, 2 dan 3 tidak menghasilkan antosianin, kultivar no. 4 menghasilkan antosianin dalam jumlah relatif kecil, sedang kultivar no. 5 dan 6 menghasilkan antosianin dalam jumlah besar. Kondisi terbaik untuk menginduksi pembentukan antosianin adalah kinetin (KIN)-Indole acetic aced (IAA) (KIN 10 ppm: IAA 0.5, 1, 3, 5, 10 ppm) di bawah pencahayaan berkesinambungan dan menggunakan petiole sebagai eksplan. Kalus hasil seleksi menghasilkan antosianin 4 kali lebih tinggi dari pada kalus tanpa seleksi. Indican dalam jumlah kecil dapat dideteksi pada ekstrak MeOH dari kalus hijau.

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INTRODUCTION

Polygonium tinchtorium Ait which is called Ai plant in Japan has been known to produce indican, an indigo precursor. Indigo is a typical Japanese stain for traditional Japanese clothes. This compound is also believed to have anti-insecticidal and anti-fungal activities. Under natural condition the resistance of plant against diseases is possitively correlated with the presence of red pigmentation of the plant. Some cultivars of this plant have red pigmentation on their stem and flower. However, wether or not, the plant resistance against disease is caused by indican or red pigment has not been fully conclusive. Nonetheless, indican and red pigment (anthocyanin) are economically important.

Tissue culture method has been exploited to study biosynthesis, biochemical and genetical regulation of secondary metabolites (Rhodes *et al,* 1989). In addition, tissue culture method has been incorporated in industry, for example, in the production of red naphtoquinone anti-inflammatory drug, shikonin by suspension culture of *Lithospermum erythrorhizon* (Tabata and Fijita, 1985).

In producing secondary metabolite through tissue culture some factors have been identified to be important. According to Zenk et al (1976) initialtissue which contains high amount of the secondary metabolite of interest must be chosen as the explant. Plant growth regulators are effective triggers of in vitro secondary metabolism, for example 5 x 10^{-5}M kinetin was effective for maximum anthocyanin synthesis in cell cultures of *Haplopappus gracilis* (Constabel, Shyluk and Gamborg, 1970). However in *Daucus carota* cell culture induction of anthocyanin synthesis took place in the medium lack of 2,4-D (Ozeki and Komamine, 1985). Selection of callus line was also proven to be an effective way of increasing secondary metabolite production.

In this experiment, the effects of explant, plant growth regulator on the anthocyanin production were evaluated. An attempt of obtaining high yielding of anthocyanin callus line by selection as well as analysis of indican content were also reported.

MATERIAL AND METHOD

Six cultivars of *Polygonum tinchtorium* Ait were studied in these experiments. Those cultivars are 1. Shouyouko shirobana, 2. Matsuae Ai, 3. Hyakkan, 4. Shouyouko Akabana, 5. Miyagi Ai and 6. Akaguki kosenbon, which are numbered 1 to 6 respectively.

Basal medium was of Murashige and Skoog (1962) consisted of NH_4NO_3, KNO_3, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, KH_2PO_4, H_3BO_3, $MnSO_4.4H_2O$, $ZnSO_4.7H_2O$, KI, $Na_2MoO_4.2H_2O$, $CuSO_45H_2O$, $CoCl_2.6H_2O$, Na_2EDTA, $FeSO_47H_2O$, Thiamin HCI, Nicotinic acid, Pyridoxin HCI, Myo inositol, solidified with 8 gr agar/l. Medium was adjusted at pH 5.6 - 5.8 with 1 N KOH or 1 N NaOH prior to autoclaving, and autoclaved at 121°C, for 15 min. The cultures were incubated the temperature of 20° C under 16 hour light period unless it is specified.

a, Callus formation

1. The effect of various plant growth regulators and light duration. Hypocotyl of

0.5 cm of 1 week old etiolated seedling were used as the explants. Those explant were inoculated in solid basal medium dispensed in plates. Each plate contained *6* explants. Ten plates were incubated in darkness while an other 10 plates were incubated under 16 hours light period. The growth regulators consisted of hours light period. The growth regulators consisted of 10-5M of kinetin, BA, IAA, NAA, 2,4-D and combination of IAA/KIN, 2. 4-D/KIN, NAA/KIN, IAA/BA, 2,4-D/BA and NAA/BA.

2. The effect of plant growth regulators on the growth of petiole explant.

Leave petioles measuring 0.5 cm of 2 - 4 months old green house grown plants were inoculated in basal medium incorporated with growth regulators. Test tubes with a rate of 1 explant per tuber were to observe for each treatment.

3. Selection for high yielding indican and anthocyanin callus line.

The reddest and the biggest calli grew on the medium containing KIN/IAA were used as explants. These petiole derived callus were subcultured to basal medium containing : (a) 10^{-5}M KIN/10^{-6}M NAA, (b) 10^{6}M KIN/10^{-6}M NAA. A rate of 6 calli pieces were inoculated in one plate. The callus cultures were incubated at 20°C, and 16 hours illumination. Two weeks after incubation half of each callus, about 0.5 g were analyzed for anthocyanin content while the other half were cut into 4 pieces and transfered to a fresh medium of the same formulation for another 2 weeks. Callus which indicated the highest anthocyanin content were then maintained for further subculture for the callus line selection. The calli of low anthocyanin content were discarded. As a control treatment initial calli grew on medium containing KIN/IAA were also subcultured and analyzed their anthocyanin content without subjected to any selection.

b. Extraction and estimation of anthocyanin content

Callus to be analyzed was taken out from plate and cleaned from agar with tissue paper and measured its fresh weight. As much as 0.1 g. f.w. callus was suspended in 2 ml of 0.1% HCI MeOH overnight, then centrifuged at 2000 rpm for 10 minutes. The supernatant was measured for its adsorbance in a cuvette of 1 cm pathlength with a UV-VIS double beam spectrophotometer; Jasco U best-30, at wavelengths ranging from 200 - 1100 nm. Absorption peak was reached at 525 nm.

c. Extraction and Estimation of Indican

Fresh Calli of 2 gram were homogenized and extracted in 15 ml cold aceton at 4°C, overnight. The extracts were subsequently filtered. The filtrates were then, evaporated to dry at 40°C under vaccuum. These dried extracts were suspended with 1 ml methanol. Then, the solutions were centrifuged at 2000 rpm for 10 minutes. The supernatants were finally injected to HPLC. Indican content was to be detected at 254 nm with Hitachi L-6000 pump, Hitachi L-4200 UV-VIS detector and Hitachi D-2500 chromato integrator. As the solvent was 5% CH_3CN in 0.1% H_3PO_4. Indican content was determined by comparing the peak of sample to the peak of indican standard.

Result and Discussion

a. Hypocotyl explant

Among the six cultivars tested, during the first week of culture of hypocotyls, the cultivars 1, 2, 3, 4 did not show any red pigmentation, the reverse was true for cultivars 5 and 6. It seems that this different is genotype dependence, since under natural condition, the stem colour of cultivars 1, 2, 3, 4 are green while of cultivars 5 and 6 show red pigmentation. Those difference in pigmentation was evident either under light or dark. However hypocotyls of cultivars 5 and 6 which were exposed to light evidently showed more pigmentation, as it was observed visually than the ones which were incubated in darkness. Light has been shown to play an important role in inducing anthocyanin synthesis (Nozue and Yasuda, 1985; Rengel and Kordan, 1987). eventhough in the case of vitis culture, light was not necessary for anthocyanin synthesis. (Yamakawa et al, 1983).

After one week of culture, calli were formed on the hypocotyl explants incubated in the medium containing (1-$^{-5}$M): 2 weeks calli were also formed from the hypocotyl explants cultured in the medium containing BA, IAA/KIN, IAA/BA.

Tabel 1. Pengaruh beberapa zat pengatur tumbuh pada pembentukan antosianin, kalus dan akar dari eksplan hipokotil dari 6 kultivar *Yolygonum tinchtorium* Ait yang tumbuh di bawa pencahayaan, sesudah 2 minggu.

Table 1. The effects of various plant growth regulators on red pigment formation, callus and root formation of six-cultivars of Yolygonum tinchtorium Ait hypocotil explants grown under light condition after 2 weeks.

Treatments (10^{-5}M)	green stem	red stem	pink	callus	root
No hormon	1,2,3,4[1] (100%)[2]	5,6 (100%)		-- (0%)	1,2 (100%)
KIN	1,2,3 (100%)	5,6 (100%)	4 (100%)	(0%)	(0%)
BA	1,2,3 (100%)	5,6 (100%)	4 (100%)	(0%)	(0%)
IA	1,2,3,4 (100%)	5,6 (100%)	(0%)	(0%)	1,2,3,4,5,6 (100%)
NAA	1,2,3,4 (100%)	5,6 (100%)	(0%)	1,2,3,4,5,6 (100%)	5,6 (50%)
2,4-D	1,2,3,4 (100%)	5,6 (100%)	(0%)	1,2,3,4,5,6 (100%)	(0%)
IAA-KIN	1,2,3,4 (100%)	5,6 (100%)	(0%)	(0%)	(0%)
NAA-KIN	1,2,3,4 (100%)	5,6 (100%)	(0%)	1,2,3,4,5,6 (100%)	(0%)
2,4-D-KIN	1,2,3,4 (100%)	5,6 (100%)	(0%)	1,2,3,4,5,6 (100%)	(0%)
IAA-BA	1,2,3,4 (100%)	5,6 (100%)	(0%)	(0%)	3,4 (100%)
NAA-BA	1,2,3,4 (100%)	5,6 (100%)	(0%)	1,2,3,4,5,6 (100%)	(0%)
2,4-D-BA	1,2,3,4 (100%)	5,6 (100%)	(0%)	1,2,3,4,5,6 (100%)	

1) Number 1,2,3, ... 6 showed number of cultivars
2) Indicates percentage of each cultivar

It's obvious that auxin was important for callus formation. Table 1 shows the response of the hypocotyl explant in culture after 2 weeks. Calli formed in this experiment were generally white. Only the one formed in the medium containing IAA/KIN showed a little pigmentation. Callus formed on the medium containing NAA was friable and showed a good appearance while callus formed on medium containing 2,4-D was compact but turned brown (indicated senescense) after 2 weeks. It is possibly because the concentration of the 2,4-D in the medium was too high.

b. Callus formation from petiole explants

Callus formation from petiole explants required the presence of auxin with or without cytokinin in the medium. In fact calli were not formed in the medium containing BA, kinetin or 4PU, but they were formed in the medium containing IAA, NAA or 2,4-D as well as in the medium containing both auxin and cytokinin.

The effects of plant growth regulators on the morphogenetic response of petiole explants could be described as follows.

Tabel 2. Kombinasi zat pengatur tumbuh terbaik untuk menginduksi kalus pada 6 kultivar *Polygonum tinchtorium* Ait.

Table 2. The best combination of plant growth regulators to induce callus in six cultivar of Polygonum tinchtorium Ait.

Cultivars		Combination of plant growth regulators
1.	Shouyouko shirobana	3 ppm BA - 0.5 ppm NAA (++++++)
2.	Matsue-Ai	3 ppm BA - 0.5 ppm NAA (++++)
3.	Hyakkan	2 ppm BA - 0.5 ppm NAA (++++++)
4.	Shouyouko Akabana	1 ppm KIN - 3 ppm 2,4-D (++++++)
5.	Miyagi Ai	3 ppm BA - 0.5; 1; 2; 3 ppm NAA 0.05 pprn KIN - 1 pprn 2,4-D (+++++)
6.	Akaguki kosenbon	1 ppm BA - 0.5 ppm NAA (+++++)

+ : showed the relative size of callus
+ : menunjukkan ukuran relatif kalus

In case of combination of KIN-2,4-D; at 2 pprn KIN, in various concentration of 2,4-D, for six cultivars, the callus size is the lowest. Callus formed at 2 pprn 2,4-D in various concentration of KIN is bigger than that at 1 pprn 2,4-D or 3 pprn 2,4-D, except for cultivar no. 4 (Kojoko Akabana). It seems that 3 pprn 2,4-D was too high to promote callus growth and 2 pprn kinetin inhibited callus growth.

In the combination of KIN-IAA, the increased of KIN concentration from 0.5 pprn to 3 pprn also caused the increased in thecallus size. Callus grown on the media containing 5 and 10 pprn KIN has the same size as the one grown in the presence of 3 pprn KIN. The increased of IAA concentration from 0.5 pprn to 10 pprn at various concentration of KIN did not give any differences in callus size. This indicates that KIN played more important role than IAA to promote callus growth.

In the case of combination of BA-NAA, in the absence of BA, in all concentration of NAA (from 0.5 ppm to 3 ppm) only small callus size formed. But when BA was added, the callus size increased, while the root formation decreased. The best combination of BA-NAA to induce callus was depent on the cultivar, as shown in table 2.

The increased concentration of 4PU in all concentration of NAA caused the increased of callus size. While the increased of NAA concentration from 0.1 ppm to 5 ppm did not cause the increased in callus size. This tendency was similar to BA-NAA tendency.

Table 2. summarizes the best growth regulators for the biggest petiole derived callus size formed in the medium. In short callus growth is depent upon the genotypes and the level of growth regulators concentration included in the medium.

c. Selection for anthocyanin high yielding callus line

Those two previous experiments significantly indicated that only cultivar no. 5 (Miyagi Ai) and cultivar no. 6 (Akaguki Kosenbon) are worth to be explored for anthocyanin production, since they showed a clear red pigment formation in all combinations of growth regulators. Furthermore, the colour formation of Miyagi Ai cultivar is controlable for some extent since it is depent upon the ratio of auxin and cytokinin (table 3). Therefore for the callus line selection experiment only callus derived from petiole explants were selected through subculture. Every subculture indicated one passage of selection.

The anthocyanin content of screened callus line was evidently increased with the passage of selection, while the content of anthocyanin of unscreened callusline remained constant. The increase of the anthocyanin content start to be constant after 12 passages (fig. 1 and fig. 2.). Miyagi Ai cultivar was of interest to be chosen as a model for callus line screening since it has been widely known. This result are true for both callus lines established in bothmedium containing 10^{-5}M kinetin/10^{-6}M NAA and 10^{-6}M kinetin/10^{-6}M NAA.

These two media were selected since callus grown on those media showed better pigmentation. The anthocyanin content of the screened callus increased up to 4 times fold after screening of 14 passages. Another interesting thing to note is that anthocyanin content of callus line grown in those two different media were also different eventhough their sizes were almost the same. However it is difficult to conclude whether the difference is simply hormon dependence or callus line variant dependence or both, inherents with the selection itself. Nonetheless the selection of callus line resulted in yielding anthocyanin callus line.

d. Indican content of selected callus line

During the course of selection, an intriguing phenomenon was observed. The screened callus, in addition to forming callus of red pigmentation, also formed callus of green colour in the same culture.

This green callus was subsequently separated and analyzed for its anthocyanin and indican content. Indican content analysis was conducted also for the red callus. The results indicated that only a little amount of indican was detected from MeOH extract of green callus (figure 3) while no indican was detected from the red callus. This result suggest that indican and anthocyanin have different biosynthetic pathway. Consequently, higher indican production may require a different biochemical trigger for cells to differentiate toward indican systhesis.

CONCLUDING REMARKS

The above experiment shows that a great variability occurs on callus culture. This variation, in one hand, can be directed for yield improvement of certain secondary metabolites as well as for biosynthesis of novel compund, but in the other hand can cause a serious problem if it is applied for large scale production of secondary metabolite. However, callus line selection can be an alternative method of improving certain secondary metabolite yield by amplyfying the target cells to get stable lines.

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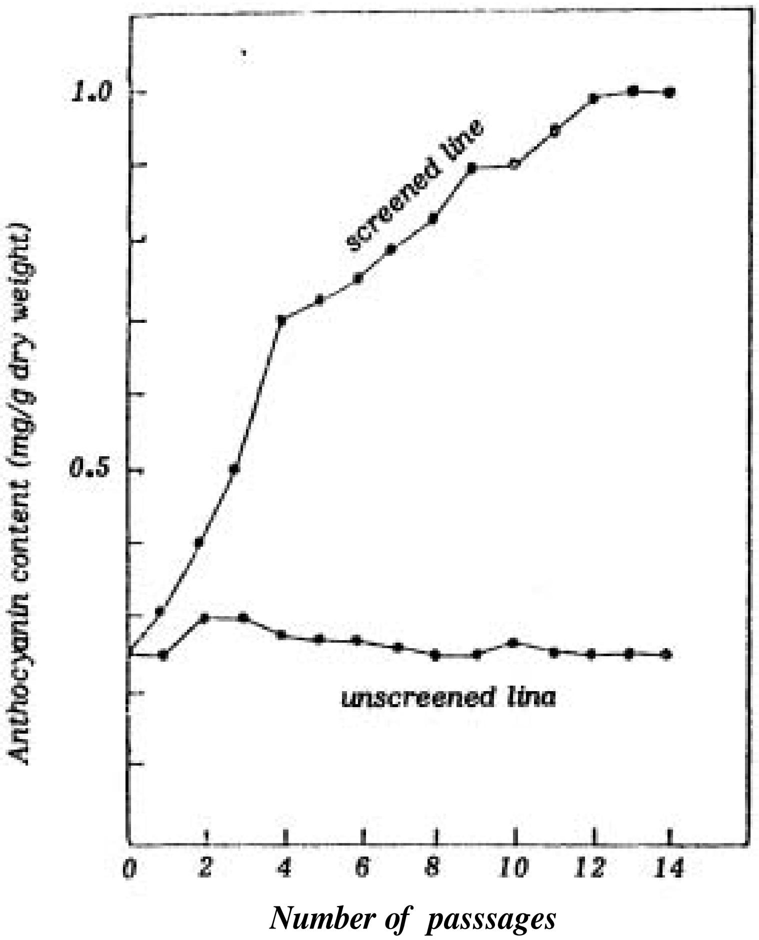
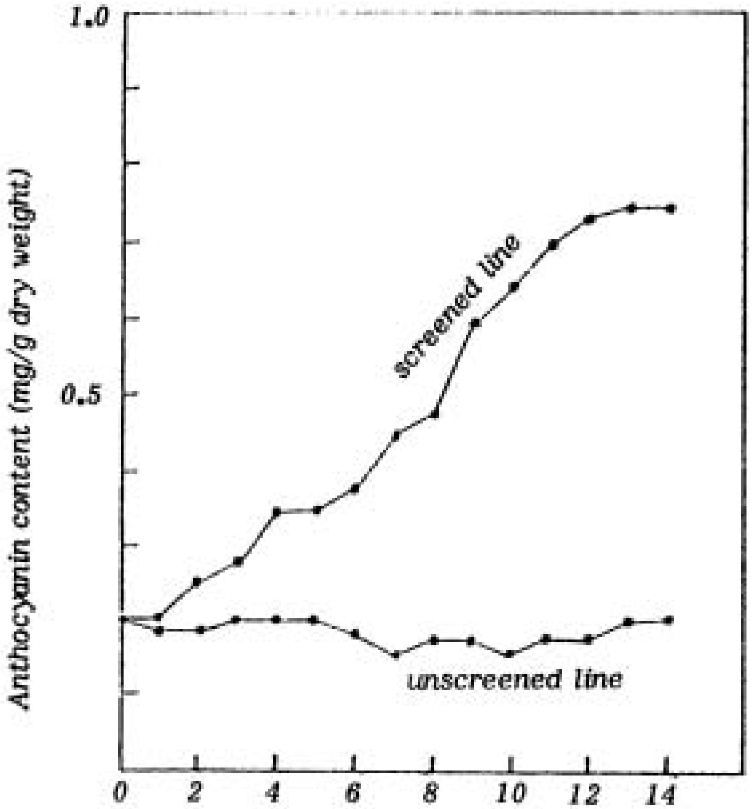


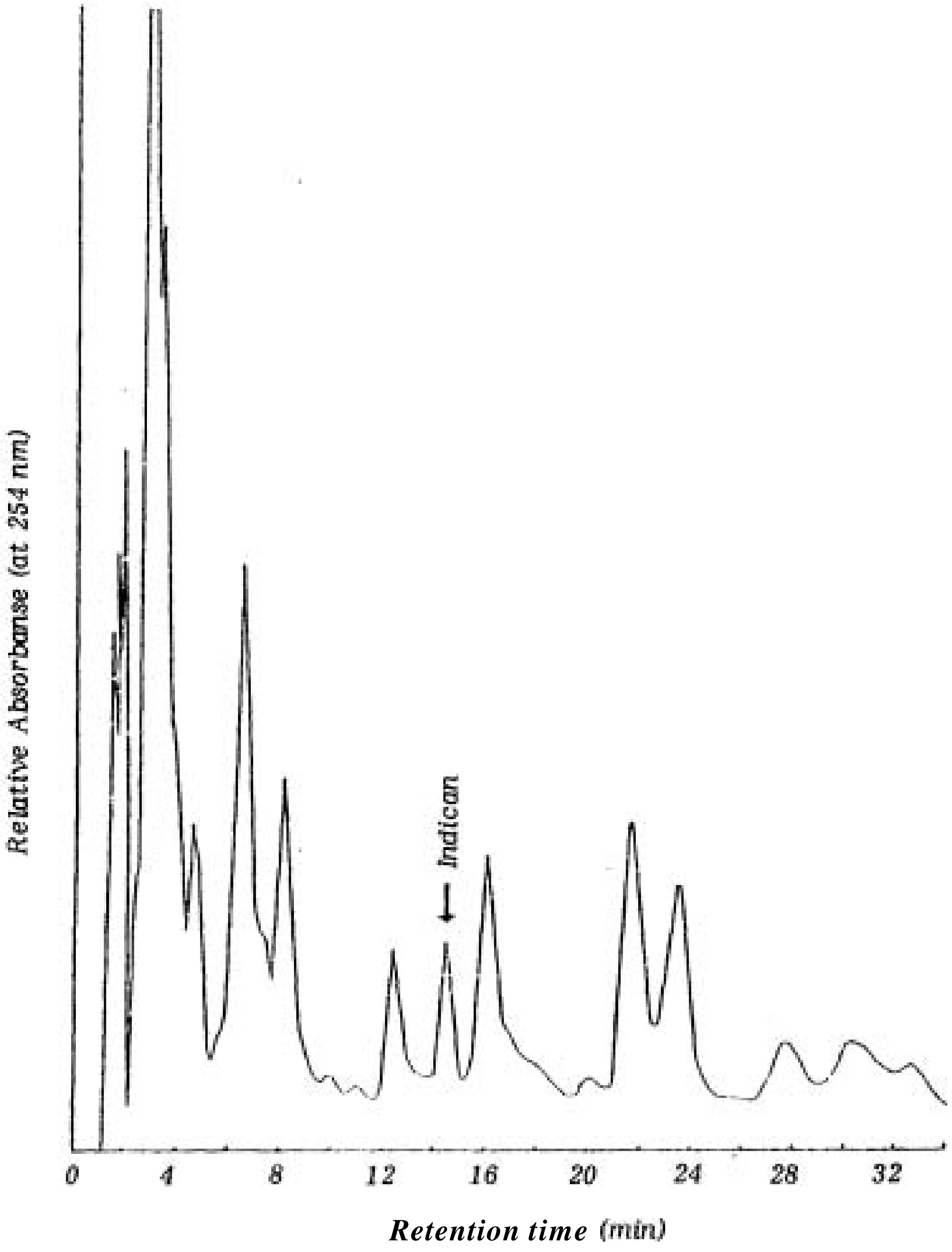
Gambar 1. Perubahan kandungan antosianin (mg/g berat kering) selama seleksi kalus yang tumbuh pada media yang mengandung KIN 10^{-5}M NAA 10^{-6}M. Data adalah rata-rata dari 3 ulangan contoh yang masing-masing dianalisa secara triplo.

Figure 1. Change in anthocyanin content (mg/g dry weight) during selection of callus grown on media containing KIN 10^{-5}M NAA 10^{-6}M. The data are the averages of triplicate samples from each of triplicate cultures, determined at the end of each passage.



Gambar 2. **Perubahan kandungan antosianin (mg/g berat kering) selama seleksi kalus yang tumbuh pada media yang mengandung KIN 10^{-6}M NAA 10^{-6} M. Data adalah rata-rata dari 3 ulangan contoh yang masing-masing dianalisa secara triplo.**

Figure 2. Change in anthocyanin content (mg/g dry weight) during selection of callus grown on media containing KIN 10^{-6}M NAA 10^{-6}M. The data are the averages of triplicate samples from each of triplicate cultures, determined at the end of each passage.



Gambar 3. Kromatografi cair kinerja tinggi fraksi yang larut MeOH dari ekstrak aceton dari kalus hijau *Yolygonum tinchtorium* Ait.

Figure 3. ***HPLC of MeOH-soluble fraction from acetone-extract of green calli of Polygonum tinctorium Ait*** *($Ch_3CN:H_2O-1:19.0.1\%H_3PO_4$).*