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Production and Characterization of an Anti-Angiogenic Agent from *Saccharomyces cerevisiae* K-7

JEONG, SEUNG-CHAN, DAE-HYOUNG LEE, AND JONG-SOO LEE*

Department of Life Science and Genetic Engineering, and Bio-Medicinal Resource Research Center, Paichai University, Daejeon 302-735, Korea

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Abstract The cell-free extracts of 250 yeasts were screened for their *in vitro* anti-angiogenic activity, to develop a new cancer metastasis inhibitor. *Saccharomyces cerevisiae* K-7 was selected as the producer of the anti-angiogenic agent, because it had the highest anti-angiogenic activity. The anti-angiogenic agent was produced maximally from hydrolysates of *Saccharomyces cerevisiae* K-7, when the yeast was cultured in yeast extract-peptone-dextrose medium at 30°C for 24 h, and cell-free extracts were than digested with pepsin for 4 h at 37°C. The anti-angiogenic agent was further purified by ultrafiltration, Sephadex G-25 gel permeation chromatography and reverse-phase HPLC, and the anti-angiogenic activity of the final purified preparation was 72.7% at 10 μM/egg. The purified anti-angiogenic agent was found to originate from the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) molecule of *Saccharomyces cerevisiae* K-7, and its peptide sequence was Val-Ser-Trp-Tyr-Asp-Asn-Glu-Tyr-Gly-Tyr-Ser-Thr-Arg-Val-Val-Asp. In the MTT assay, the shape of the HT-1080 cell was clearly changed to a circular type at 0.2 mM purified anti-angiogenic agent. This result indicated that the growth of the HT-1080 cell was significantly inhibited at 0.2 mM of the purified anti-angiogenic agent. The MMP activity of the treated HT-1080 cells was not affected, evidenced by the gelatin zymography, indicating that the anti-angiogenic mechanism of the purified anti-angiogenic agent is not mediated through MMP activity.

Key words: *Saccharomyces cerevisiae* K-7, anti-angiogenic agent, cancer metastasis inhibitor, bioactive peptide

Angiogenesis is a complex process involving a cascade of events that result in the formation of new blood vessels. Such vessel formation takes place under physiological conditions, such as embryogenesis, ovulation, and wound healing, as well as pathological ones, including tumor neovascularization and retinopathies [6, 31, 40]. In particular, it is important for the proliferation and metastasis of solid tumors [7, 12]. After capillarization, tumors grow rapidly into large masses [17]. Extensive experimental and clinical data support the concept that the growth of tumors greater than 1 to 2 mm^2 is critically dependent on angiogenesis [8, 10]. Importantly, the process of tumor neovascularization is induced by tumors rather than being a passive dilatation of the preexisting vessels [1, 9]. A number of molecules produced by tumor cells have been implicated as angiogenesis mediators for tumors [9] and some growth factors [14, 42], inflammatory cytokines (*e.g.*, tumor necrosis factor-α and interleukin-8 [11, 33]), and angiogenin [5] are also known to promote tumor angiogenesis. In tumor-associated angiogenesis, activated endothelial cells from pre-existing venule invade the stroma through the basement membrane of the parent vessel, and migration of the advancing tip is directed toward an angiogenesis stimulus by the tumors. Sprout elongation is supported by the proliferation of proximal endothelial cells. After formation of a lumen and perfusion of two neighboring sprouts, blood perfusion starts in the newly originated capillary loop. A growing number of diseases, such as solid tumors and metastasis, hemangiomas, diabetic retinopathy, psoriasis, neovascular glaucoma, and rheumatoid arthritis, are characterized by the pathological growth of new capillaries and are now considered to be "angiogenic diseases" [39]. Some small molecules such as gentisyl alcohol also induce angiogenesis as a potential therapy of ischemia-related diseases [23]. Therefore, the availability of a chemical agent or Chinese herbal medicine that could prevent the continued spread of vascularization would have potentially broad applicability

*Corresponding author
Phone: 82-42-520-5388; Fax: 82-42-520-5388;
E-mail: biotech8@pcu.ac.kr

as a therapy for those diseases in which angiogenesis plays an important role. Thus, anti-angiogenic agents might be a novel strategy for tumor growth inhibition.

Many anti-angiogenic agents such as angiostatin, endostatin, heparin, ursolic acid, squalamine, interferon-α and neovastat have been reported. Moreover, new peptides and antibodies with anti-angiogenic activity are currently being tested in clinical trials for their therapeutic efficacy [4, 29]. However, commercial anti-angiogenic drugs have some disadvantages such as high cost, low yield, ineffectiveness, and side effects *in vivo*. For those reasons, this study was carried out to investigate the production of a novel anti-angiogenic agent from yeast for the purpose of developing a new anticancer metastasis drug or nutraceutical. The anti-angiogenic agent was purified from yeast and characterized. Furthermore, the anticancer metastasis function of the anti-angiogenic agent was determined by animals testing.

MATERIALS AND METHODS

Yeast Strains and Human Cells

Several *Meju* yeasts [38], industrial yeasts, and other yeasts were obtained from the Laboratory of Biotechnology, Paichai University, Korea Culture Center of Microorganism (KCCM), and Korea Collection for Types Cultures (KCTC).

HT-1080 human fibrosarcoma (ATCC CLL-121) cells were used in this study. The human fibrosarcoma HT-1080 cell line was maintained in a culture medium consisting of M199 medium (GIBCO BRL), supplemented with 10% fetal calf serum (GIBCO BRL), in a humidified atmosphere of 5% CO_2 at 37°C.

Materials

Unless otherwise specified, all chemicals and solvents were of analytical grade. Sephadex G-25 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Fertilized chicken eggs were obtained from Hyong-Je Farm (Daejeon, Korea). Fat emulsion (10%) was acquired from Green Cross Pharm. Co. (Seoul, Korea). Thermanox coverslips were purchased from Nunc Inc. (Naperville, IL, U.S.A.). Acetonitrile water for HPLC was obtained from J. T. Baker (U.S.A.).

Screening of the Anti-Angiogenic Agent-Producing Yeast

Yeasts from several sources were inoculated in 20 ml of YEPD medium and cultured for 72 h at 30°C. After centrifugation at 15,000 ×*g* for 15 min, supernatants and cells were obtained. The anti-angiogenic activities in the supernatants were determined in order to select the extracellular anti-angiogenic agent-producing microorganism. The cells were suspended in 5 ml of phosphate buffer (pH 7.0), washed, resuspended in the phosphate buffer (pH 7.0), and then disrupted by a glass bead. After centrifugation at 15,000 ×*g* and 4°C for 10 min, the anti-angiogenic activities in the supernatants were determined.

Assay for Anti-Angiogenic Activity

Anti-angiogenic activity was determined by chorioallantoic membrane (CAM) assay with a modification of the method of Okikawa *et al.* [41] and Crum *et al.* [3, 35]. Fertilized chicken eggs were used in this study, and kept in a humidified egg incubator at 37°C. After three days of incubation, approximately 5 ml of albumin was aspirated from the eggs with an 18-gauge hypodermic needle through a small hole drilled at the narrow end of the egg, allowing the small CAM and yolk sac to drop away from the shell membrane. On day 4, the shell covering the air sac was punched out and removed with forceps, and the shell membrane on the floor of the air sac was peeled away [39]. Embryos with chorioallantois of 3–5 mm in diameter were employed for the assay of the anti-angiogenic activity. Thermanox coverslips were loaded with various doses of samples. After air-drying, the loaded coverslips were then applied to the CAM surface of 4.5-day-old chick embryos. Three days later, an appropriate volume of 10% fat emulsion (10% Intralipid, Green Cross Co. Korea) was injected into the 6.5-day-old embryo chorioallantois, and the neovasculature was observed under a microscope. The anti-angiogenic response was assessed by measuring an avascular zone of the CAM beneath the disk. When the CAM showed an avascular zone of 3 mm or larger in diameter, the response was scored as positive according to the method of Crum *et al.* [3].

MTT Assay for Determination of HT-1080 Cancer Cell Growth

The effects of a purified anti-angiogenic agent on the growth of HT-1080 cancer cells was assessed using the MTT assay, which is based on the conversion of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] to MTT-formazan by mitochondrial enzyme [44]. In brief, the HT-1080 cells were plated in a 96-well plate at a density of 2×10^3 cells per well and were cultured with 200 µl of complete medium per well for 24 h at 37°C under 5% CO_2. Then, the cells were treated with the desired concentrations (25 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml, and 400 µg/ml) of purified angiogenic inhibitor-conditioned medium for 48 h. After the freshly treated media were changed, cells were incubated for a further 24 h. Five mg/ml of the MTT solution was added to the incubation mixture and incubated for another 4 h. Subsequently, 100 µl of the supernatant was added to 100 µl of DMSO. Absorbance of each well was read at 540 nm with a micro-ELISA reader (Molecular Devices, Sunnyvale, CA, U.S.A.). The percent of cell survival was defined as the relative absorbance of treated versus untreated cells.

Gelatin Zymography

Both the control and treated cells were incubated for 48 h in serum-free media. After incubation, supernatants were collected, then centrifuged to eliminate cell debris, and the total protein content was determined (Berkman, DU650). The zymography assay was performed as described in the MMP activity analysis with a slight modification: 10% SDS-PAGE gels were co-polymerized with 0.1% gelatin and the samples were electrophoresed. After electrophoresis, the gels were washed twice in 2.5% triton X-100 to remove SDS and incubated for 18 h at 37°C in the solution containing 40 mM Tris, 200 mM NaCl, and 10 mM $CaCl_2$, pH 7.5, which permits the enzymatic activity to proceed. After staining the gels with 0.1% Coomassie brilliant blue, areas of lysis were observed as white bands against a blue background.

Preparation of Protein Hydrolysate

To increase the anti-angiogenic activity, the pH of cell-free extracts of *S. cerevisiae* K-7 was adjusted to an optimum pH of each protease and digested with 1% (w/v) each of pepsin (37°C), trypsin (25°C), and protease N (55°C) at an optimum temperature for 12 h. The reaction was terminated by heating the extracts in boiling water for 10 min, and the precipitate was separated by centrifugation. The dissolved solution in 20 mM phosphate buffer (1 ml) was used as the protein hydrolysate.

Purification of Anti-Angiogenic Agent

The protein hydrolysate of *S. cerevisiae* was ultrafiltrated with 5,000 Da cut-off filter (Labscale TFF System, Millipore Co., U.S.A.), and the anti-angiogenic activity of the filtrates was than determined. The active fraction was concentrated by lyophilization and then applied to a Sephadex G-25 column (3.0×35 cm) equilibrated with distilled water, and the column was eluted with the same buffer at a flow rate of 12 ml/h. The fractions with anti-angiogenic activity were then applied to a preparative reverse-phase high-permeation liquid chromatography (μBondapak C_{18} column) equilibrated with acetonitrile [34]. A linear gradient formed with 0.1% trifluoroacetic acid (TFA) from 0 to 100% (v/v) in water was used to elute the column. The active fractions were collected and lyophilized immediately.

Determination of Molecular Weight and Amino Acid Sequence by Mass Spectrometry

The molecular mass of the purified anti-angiogenic agent was determined using an LC/MS spectrophotometer (HP 1100 series LC/MSD, U.S.A.). The amino acid sequence was determined by the method of Edman using an Applied Biosystems 491A automatic protein sequencer [46].

RESULTS

Screening of the Anti-Angiogenic Agent-Producing Yeast

To select the anti-angiogenic agent-producing yeast, 250 kinds of yeasts were tested for their anti-angiogenic activities. The cell-free extract of *S. cerevisiae* K-7 (Kyokai No. 7) showed the highest anti-angiogenic activity of 46% (Table 1). Therefore, we finally selected *S. cerevisiae* K-7 as the producer of a new intracellular anti-angiogenic agent. *S. cerevisiae* K-7 has long been used in the brewing of some alcohol beverages such as Korean rice wines (*Yakju*, *Tagju*, and traditional rice wine) [25, 26, 35] and sake [45], because of its high ethanol productivity, and we also reported previously on the production and characterization of a novel antihypertensive angiotensin I-converting enzyme (ACE) inhibitor from *S. cerevisiae* [24]. Even though some bioactive compounds were identified and characterized recently from edible fungi [15, 21, 24, 27, 34, 37] and some Korean traditional fermentative foods [25, 36, 42], this is the first report that alcohol fermentative *S. cerevisiae* K-7 produced a potent intracellular anti-angiogenic agent. This could have an important application in several fields including the food and medicinal industries.

Table 1. Anti-angiogenic activities of the secondary selected yeasts.

Yeast strains	Anti-angiogenic activity (%)
Candida tropicalis KCTC 7725[a]	30.0±1.3[b]
Pichia anomala KCCM 11473	12.5±2.0
Pichia membranaeifaciens KCTC 7628	30.0±1.9
Rhodotorula glutinis KCTC 7693	10.0±3.1
Saccharomyces cerevisiae K-7 (Kyokai No. 7)	46.0±2.4
Saccharomyces diastaticus KCTC 7110	11.0±1.5
Saccharomyces pastorianus KCTC 7918	12.0±2.0
Kluyveromyces lactis var. *lactis* KCTC 7138	25.0±1.5
Zygosaccharomyces mellis KCTC 17270	10.5±1.8
Zygosaccharomyces rouxii KCTC 7191	13.5±2.2

[a]Yeasts that showed up to 10% of anti-angiogenic activity in secondary screening tests.
[b]Means±SD. This experiment was repeated three separate times.

Table 2. Effect of proteases on anti-angiogenic activity of cell-free extracts from *S. cerevisiae* K-7.

Anti-angiogenic activity (%)			
Control	Protease N	Trypsin	Pepsin
48.0±1.5	55.0±2.1	47.0±0.8	66.7±1.7

Culture conditions to produce the anti-angiogenic agent from *S. cerevisiae* K-7 were investigated. The maximum cell growth was reached at 48 h of cultivation, whereas the maximum production of the anti-angiogenic agent was achieved at 24 h of cultivation. The optimal culture temperature for the production of the anti-angiogenic agent was 30°C.

Generally, many bioactive compounds from yeasts, including antihypertensive angiotensin I-converting enzyme(ACE) inhibitor and anti-angiogenic agents, are known to be peptides [24]. To increase the productivity of the anti-angiogenic agent, cell-free extracts of *S. cerevisiae* K-7 were treated by various proteases under each optimal reaction condition, and the anti-angiogenic activities were determined. As shown in Table 2, the anti-angiogenic activity increased approximately 1.5-fold (66.7%) by treatment of cell-free extracts with pepsin.

Purification of the Intracellular Anti-Angiogenic Agent from *S. cerevisiae* K-7

To investigate the physicochemical properties of the anti-angiogenic agent and to further elucidate the structure-function relationship, the anti-angiogenic agent in the pepsin-hydrolysates of the cell-free extracts of *S. cerevisiae* was purified as described in Material and Methods.

The anti-angiogenic activity of the filtrates from 5,000 Da cut-off ultrafiltration of the pepsin hydrolysate was determined to be 68.9%. After Sephadex G-25 column chromatography, the active fraction showed 70.0% of the anti-angiogenic activity. Active fractions from the above column chromatography were pooled, and preparative reverse-phase HPLC was performed by using the μBondapack C_{18} column following repeated RP-HPLC. One peak containing anti-angiogenic activity was finally obtained (Fig. 1), and its anti-angiogenic activity was 72.7%/μg, which was increased about 15-fold from that of the cell-free extracts, and its solid yield was 1.5% (data not shown).

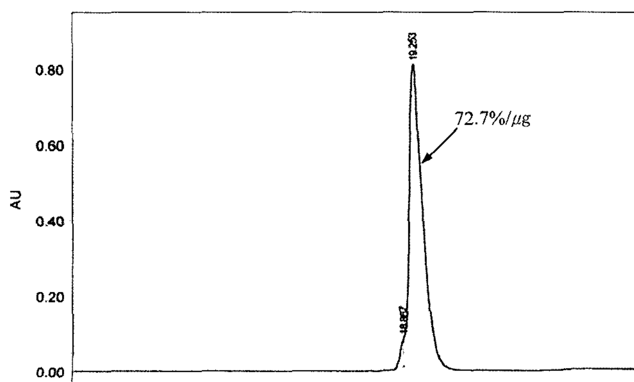


Fig. 1. Reverse-phase HPLC profile in μBondapak C_{18} column chromatography.

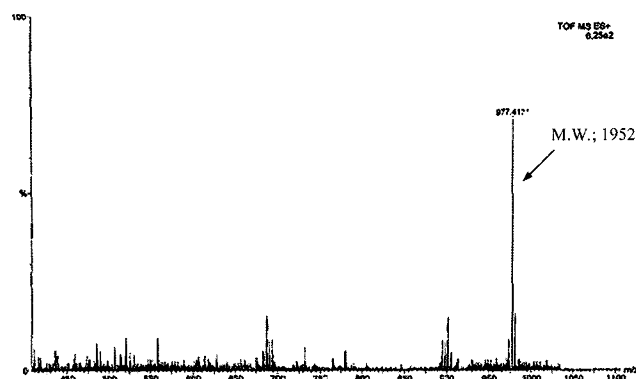


Fig. 2. LC-MS spectrum of the purified anti-angiogenic agent from *S. cerevisiae* K-7.

Characteristics of the Purified Anti-Angiogenic Agent

The molecular mass of the anti-angiogenic agent was estimated to be 1,952 daltons using an LC-MS analysis (Fig. 2), and its amino acid sequence was found to be Val-Ser-Trp-Tyr-Asp-Asn-Glu-Tyr-Gly-Tyr-Ser-Thr-Arg-Val-Val-Asp by a tandem LC-MS analysis (Fig. 3). Even though its molecular weight was larger than those of the other nutraceuticals [24, 35], the anti-angiogenic agent was isolated for the first time from *S. cerevisiae,* and showed a unique sequence without any homology. According to the BLAST homology search test, this peptide sequence was originated from glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (No. 309–324) in *S. cerevisiae.* (http://www.ncbi.nlm.nih.gov). HT-1080 (ATCC CLL-121), an established cell line from human fibrosarcoma cells, was used as test cells. The effect of the purified anti-angiogenic agent on the proliferation of HT-1080 cells was investigated with the MTT assay, after the cells were seeded at 2.0×10^3 cell/well, and various concentrations of purified anti-angiogenic agent were added. The shape of the HT-1080 cells in the presence of 400 μg/ml purified anti-angiogenic agent was clearly changed to a circular type (Figs. 4 and 5). This result indicates that the growth of HT-1080 cells was significantly inhibited at 0.2 mM of the purified anti-angiogenic agent. Therefore, the purified anti-angiogenic agent is expected to be effective in preventing proliferation of cancer cells, and gelatin zymography revealed that the purified anti-angiogenic agent did not affect the MMP activity of HT-1080 cells (data not shown).

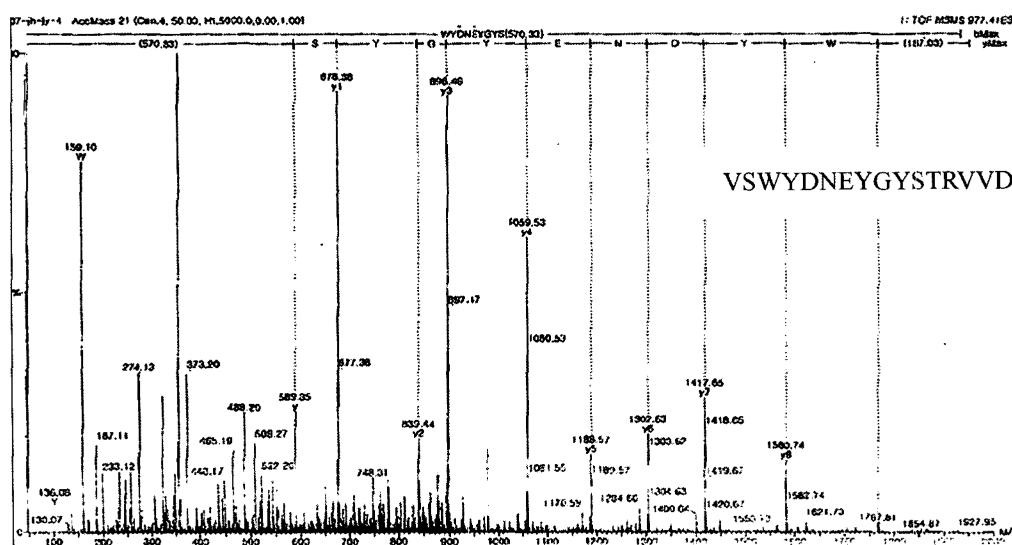


Fig. 3. The peptide sequence of the anti-angiogenic agent from *S. cerevisiae* K-7, determined by tandem LC-MS analysis.

DISCUSSION

The purpose of this study was to produce, purify and characterize the anti-angiogenic agent from edible and alcohol fermentative *S. cerevisiae* K-7 in order to develop a new anticancer metastasis agent or nutraceutical. We finally selected *S. cerevisiae* K-7, with high anti-angiogenic activity of 46%, by using CAM assay of several yeasts extracts. Several kinds of anti-angiogenic agents have been isolated from many plants or herbs, such as resveratrol from grape [2], phenolic compounds from poplar leaves [13], shikinin from *Lithospermum erythrorhizon* [16], epigallocatechin gallate (EGCG) from green tea [18], sesquiterpene from *Torilis japonica* [26], deoxypodophyllotoxin from *Pulsatilla koreana* [30], aqueous extracts from *Berberis paraspecta*, *Catharanthus roseus*, *Coptis chinensis*, *Taxus chinensis*, *Scutellaria baicalensis*, *Polygonum cuspidatum*, and *Scrophularia ningpoensis* [47], and isoliquiritin from *Licorice* root [32]. Moreover, Jung *et al.* [18, 19] reported that human endostatin at 1×10^{-4} M/l (2 mg/ml) significantly inhibited the initiation rate of an angiogenic response in human blood vessels *in vitro*, whereas human angiostatin did not inhibit the initiation of an angiogenic response. Furthermore, Kim *et al.* [22] also reported that the synthetic nonapeptide NSAVLQVEN (NSA9) suppressed angiogenesis in chicken embryos. However, little study has been carried out on anti-angiogenic agents from microorganisms. The *S. cerevisiae* K-7 in this study is the first yeast known to be the producer of a new intracellular anti-angiogenic agent. *S. cerevisiae* K-7 has been used in the brewing of several Korean traditional wines, because of its high ethanol productivity [25, 26, 35], and we recently reported that the yeast produced an antihypertensive angiotensin I-converting enzyme inhibitor [24]. Therefore, it seems to be possible to directly prepare functional or medicinal foods without any side effects.

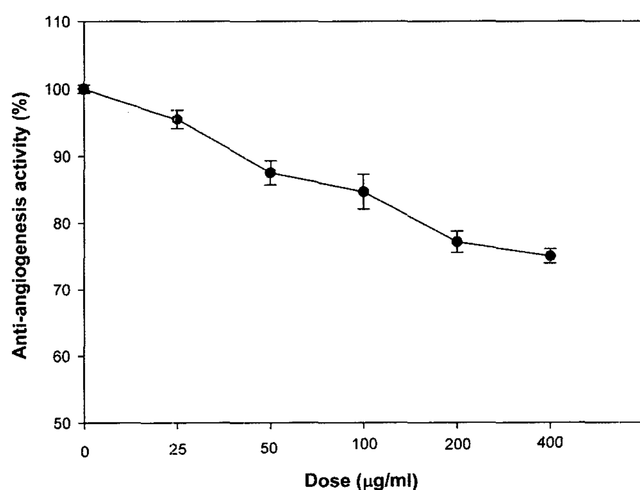
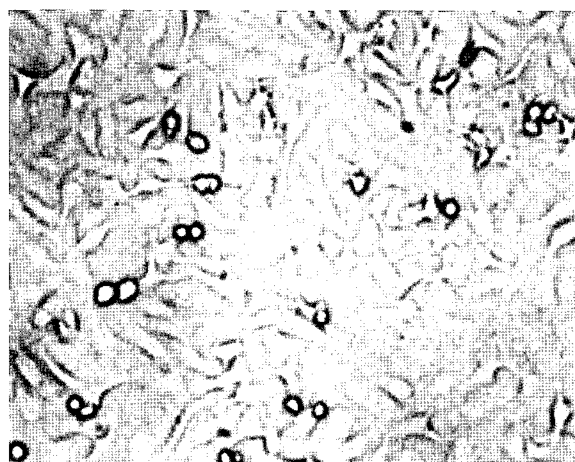


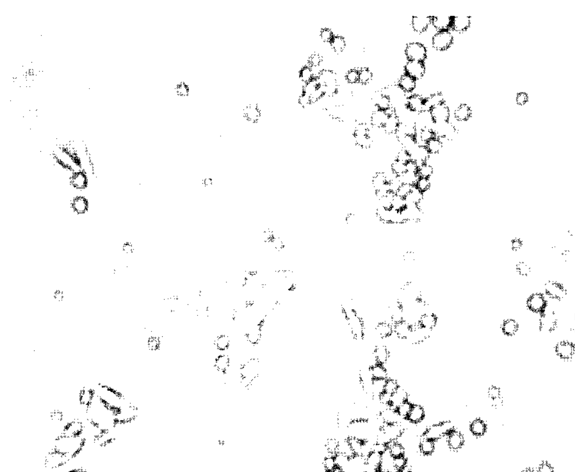
Fig. 4. MTT assay of HT-1080 after treatment with the purified anti-angiogenic agent.

Its anti-angiogenic activity was increased approximately 1.5-fold by treatment of cell-free extracts (66.7%) with pepsin. This means that the anti-angiogenic agent from *S. cerevisiae* may be free peptides or peptides generated by pepsin hydrolysis.

The anti-angiogenic agent from *S. cerevisiae* K-7 was purified by ultrafiltration, Sephadex G-25 gel permeation chromatography and reverse-phase HPLC, and a purified anti-angiogenic agent with an inhibitory activity of 72.7% per μg was obtained. The anti-angiogenic activity was stronger than that of a commercial retinoic acid (10 μg/egg). The molecular weight of the purified anti-angiogenic agent was estimated to be 1,952 daltons, and its amino acid



Control



Treatment (400 μg/ml)

Fig. 5. Photomicrography of HT-1080 cell proliferation after treatment with the purified anti-angiogenic agent.

sequence was found to be Val-Ser-Trp-Tyr-Asp-Asn-Glu-Tyr-Gly-Tyr-Ser-Thr-Arg-Val-Val-Asp. Evidence indicated that the anti-angiogenic peptide was originated from GAPDH in *S. cerevisiae* K-7. This result indicates that the anti-angiogenic peptide was endogenously produced as peptides from pepsin hydrolysis or free peptides. Although the molecular weight of the anti-angiogenic agent was larger and its peptide sequence was also longer than that of the antihypertensive compounds from the same strain as *S. cerevisiae* [24], the presently described anti-angiogenic agent is the first isolated from edible *S. cerevisiae* K-7, and has a unique sequence without any homology.

In the MTT assay using HT-1080 human fibrosarcoma cells, 0.2 mM purified anti-angiogenic agent inhibited the growth of HT-1080 cell significantly, suggesting that the purified anti-angiogenic agent should be effective in preventing proliferation of cancer cells. Based on the results of the MMP activity assay of treated cells, we concluded that the anti-angiogenic activity of the agent was not mediated through MMP activity. Further studies on the mechanism of the anti-angiogenic agent are required.

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