COMPONENTS ISOLATED FROM SAIKO ROOT (*BUPLEURI RADIX*) BIND TO CHOLECYSTOKININ TYPE-B RECEPTOR

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ABSTRACT

In the present study, we have isolated and purified chemical components in Saiko root (*Bupleuri radix*) extracts having potent binding affinity to cholecystokinin type-B (CCK-B) receptor. Radio receptor assay for CCK-B receptor was successfully used for detection of the active components throughout the purification steps. Saiko root was extracted with distilled water, ethanol, acetone and hydrochloric acid, respectively. Among the extracts, the water extract exhibited the highest binding activity for CCK-B receptor in rat cerebral cortex and GH₃ cells. The water extract was then used as the starting material. Isolation of the active component was performed by (1) gel filtration on Sephadex G-50, (2) ion exchange chromatography on a Q-Sepharose column, and (3) reverse phase HPLC on a YMC D-ODS column. Finally, the active fraction having a strong binding affinity for CCK-B receptor was purified by RP-HPLC to give a single sharp peak eluted at 17 min. These results suggest that the substance isolated in the present study may be a peptide. In fact, the sequence analysis of this substance revealed that the N-terminal sequence was Ser-Tyr-Arg-Glu-Pro-Asn-Thr-.

Saiko (*Bupleuri radix*) is a well-known Chinese herbal medicine and extensively prescribed in the Chinese clinical practice. Traditionally, the prescription which composed of Saiko root as a main crude herb has been considered to be effective in the treatment of gastrointestinal complaints, hepatocystic disorder, and psychosomatic disease. Among the components in Saiko root, Saiko saponins have been shown to exhibit effects on membrane fluidity (1), stimulation of the pituitary-adrenocortical axis (5), stimulation of enzymatic activities in liver and the inhibitory action against hepatic injury (2). However, there is little systematic study concerning the biochemical aspects of the components of Saiko roots. Chinese medicine containing Saiko root is generally administered orally to patient, absorbed in the gastrointestinal tract, and then transferred to blood circulation to exert various pharmacological actions. It is reasonable to consider that the effects of these herbs

might be in part due to the stimulation of neuro-endocrine system in the gut. In our previous study, administration of Daisaikoto (Saiko-containing medicine) exhibited a remarkable pharmacological action to stimulate the release of gastrin, secretin and insulin in rats (11). In the present study, we first purified components in Saiko root having a strong binding activity to cholecystokinin type B (CCK-B) receptor. The present finding would be valuable for exploring biological or pharmacological effects of traditional Chinese medicines containing Saiko root.

MATERIALS AND METHODS

Extraction of Saiko Root

Saiko roots were supplied by Yamamoto & Co. (Tokyo, Japan). Roots were cut into small pieces and homogenized with 5- to 10-fold vol of distilled water, 50% ethanol, acetone or 0.01 N hydrochloric

acid, respectively. Each one of these homogenates was filtered through a filter paper (No. 2, Advantec Toyo Inc., Tokyo, Japan) under reduced pressure. The solution was centrifuged at $15,000 \times g$ for 25 min, and the supernatant was lyophilized and then stored at -20°C until use.

Receptor Binding Assay

Receptor binding assay (RRA) was carried out in the manner as described previously (16). Briefly, the membrane of rat cerebral cortex was prepared by homogenization in 10 vol of 50 mM Tris HCl buffer (pH 7.4 at 4°C) containing 5 mM MgCl_2 , 0.1% bacitracin and 0.4% bovine serum albumin. Homogenates were centrifuged at $50,000 \times g$ for 20 min, and pellets were resuspended in the buffer and centrifuged as above. The membrane pellets obtained were used for binding assay. The membrane (approximately 1 mg protein) was preincubated in the same buffer for 15 min at 25°C . [^3H]Propionylated CCK-8 ([^3H]CCK-8) (Amersham Life Science, England; 65–85 Ci/mmol) was added to a final concentration of 1 nM, and the mixture was then incubated for 60 min at 25°C with increasing concentration of unlabeled CCK-8 or Saiko root extracts which were diluted with the same buffer as described above. Nonspecific binding was defined as the radioactivity remaining in the presence of 1 μM CCK-8. The binding reaction was terminated by adding 1 ml of the ice-cold buffer used for homogenization. The mixture was rapidly filtered through a Whatman GF/B glass filter. The filter was washed three times with 3 ml of the same buffer. The radioactivity retained on the filter was counted by a liquid scintillation counter (LSC-900, Aloka Co., Japan).

The binding experiments of Saiko root extract to CCK-B receptor on GH_3 cells (donated by Professor K. Inoue, Gunma University) were performed according to the method of Kuwahara *et al.* (8), with slight modifications. Briefly, GH_3 cells were cultured in Ham's F-10 medium supplemented with 2.5% fetal calf serum and 15% horse serum for 6–7 days prior to use. The cells were scraped with Dulbecco's phosphate buffer solution containing 0.02% EDTA, and washed three times with incubation buffer (Ham's F-10 medium containing bacitracin and 20 mM HEPES, pH 7.6). The cells were resuspended in the same buffer at a density of 2×10^6 cells/ml, incubated with [^3H]CCK-8 and the water extract of Saiko root at 37°C for 60 min. The binding reaction was terminated by adding 1 ml of

ice-cold saline and then counted for radioactivity.

Gel Filtration of Saiko Root Extract

The water extract of Saiko root (3 g) was dissolved in 0.01 M NH_4HCO_3 (15 ml), and the solution was applied to a column (1.5×130 cm) of Sephadex G-50 superfine (Pharmacia Fine Chemicals, Uppsala, Sweden), which was eluted with 0.01 M NH_4HCO_3 containing 0.02% sodium azide, and fractions of 4 ml were collected. The absorbance of the fractions was monitored at 280 nm, and aliquots of each fraction were used for CCK receptor binding assay. Based on the elution profile of binding activity for CCK receptor, fractions 25 to 39, 40 to 50 and 67 to 83 were collected and lyophilized, separately. These fractions were called as fraction A-1, A-2 and A-3, respectively.

Ion Exchange Chromatography

The fraction A-3, having the highest binding activity was dissolved in 10 mM Tris-HCl (pH 7.4), and insoluble materials were removed by centrifugation. The supernatant was applied to a Q-Sepharose fast flow column (0.5×10 cm, Pharmacia, Uppsala, Sweden) equipped with Waters 625 LC system (Waters Chromatography Division, Millipore, MA, U.S.A.). The column was equilibrated with the starting buffer (10 mM Tris-HCl, pH 7.4) and eluted with a linear gradient of NaCl from 0 to 1 M in 10 mM Tris-HCl (pH 7.4) for 25 min. The flow rate was 1 ml/min. The fractions (1 ml each) were collected and assayed by RRA. The main fractions having binding activity (fractions 25 to 29) were pooled. These fractions were called as fraction B-1. The fraction B-1 was then desalted on a Sephadex G-50 superfine column. The active fractions were pooled and lyophilized.

Reverse-Phase HPLC

The lyophilized fraction B-1 was dissolved in 0.01 N HCl, and applied to a column of YMC-pack D-ODS-5-A (20×250 mm I. D., YMC, Kyoto, Japan) equipped with Waters 625 LC system, which was eluted with a linear gradient of acetonitrile from 15% to 50% in 0.01 N HCl for 35 min at a flow rate of 5 ml/min, and each fraction was monitored at 210 nm.

The peak fraction (fraction C-1) thus obtained was lyophilized, and then re-chromatographed on a column of YMC-pack D-ODS (4.6×250 mm,

Fig. 1 Extraction efficiencies of inhibiting substance from Saiko root. ○—○, water extract; ▲---▲, ethanol extract; ●·····●, HCl extract; △---△, acetone extract

YMC, Kyotō, Japan). The purified fraction (fraction D-1) was lyophilized and used for sequencing.

Analysis of Amino Acid Sequence

Peptide in the fraction D-1 was sequenced by automated Edman degradation, using an Applied Biosystems model 477A Protein Sequencer equipped with an on-line PTH Analyzer (model 120A).

RESULTS

Binding Activity of Saiko Extracts

Binding activity of Saiko extracts was measured by monitoring the competitive inhibitory activity against [3 H]CCK-8 binding to CCK-B receptors. All the extracts of Saiko root with various solvents were found to inhibit the binding of [3 H]CCK-8 to CCK-B receptor on the rat cerebral cortex membrane. However, much higher binding activity was found in the water extract, compared to extracts with other solvents examined. For example, 10 μ g of water extract of Saiko root showed 38% inhibition, while the ethanol extract showed 23% inhibitory activity. In addition, the water extract inhibited [3 H]CCK-8 binding to the rat cerebral cortex membrane in a dose-dependent manner (Fig. 1).

GH₃ cells, a rat anterior pituitary tumor cell line, have been known to express CCK-B receptor (8, 15). Using the GH₃ cells, the binding activity of the water extract to CCK-B receptor was examined. The result shows that the water extract

exhibits inhibitory activity for [3 H]CCK-8 binding on GH₃ cells in a dose-dependent manner, which was similar in magnitude as that to CCK-B receptor on the rat cerebral cortex membrane (Fig. 2).

These results indicate that the water extract of Saiko root inhibits competitively [3 H]CCK-8 binding to CCK-B receptors in both cerebral cortex membrane and GH₃ cells, suggesting that Saiko root contains components that may act as a CCK agonist or antagonist. The water extract was then subjected to the following purification steps.

Gel Filtration of the Water Extract of Saiko Root

The water extract (3 g/15 ml) was subjected to gel filtration on a Sephadex G-50 column to separate the active components. As shown in Fig. 3, the elution profile of binding activity for CCK-B receptors showed three main peaks. The active fractions were grouped into the fraction A-1 (fractions 25 to 39), A-2 (fractions 40 to 50) and A-3 (fractions 67 to 83). The weight of lyophilized materials of A-1, A-2 and A-3 was 0.19 g, 0.122 g and 1.074 g.

Based on the dose responses of fractions A-1, A-2 and A-3, the inhibitory binding activity of A-3 was found to be most potent, when compared with that of A-1 or A-2. At a dose of 1 μ g, fraction A-3 exhibited the inhibition higher than 45%, while the fractions A-1 and A-2 exhibited lower inhibitory activity.

These results strongly suggest that the most

Fig. 2 Inhibitory activity of the water extract in Saiko root for [^3H]CCK-8 binding to CCK-B receptor on GH₃ cells

Fig. 3 Inhibitory activity of gel-filtration fractions on CCK-B receptor binding assay. Water extract of Saiko root (3 g) was dissolved in 0.01 M NH_4HCO_3 (15 ml), and applied to a column (1.5 $\times$ 130 cm) of Sephadex G-50, which was eluted with 0.01 M NH_4HCO_3 . ●—●, inhibitory activity; ○—○, absorbance

potent active components are contained in the low molecular weight fraction (A-3). Thus the fraction A-3 was further purified by gel filtration on the same column. As shown in Fig. 4, the major active fractions were again eluted in a low molecular weight region. These fractions were lyophilized and further purified by ion exchange chromatography.

Ion Exchange Chromatography

The fraction A-3 was subjected to ion exchange chromatography on a Q-Sepharose fast flow column. Fig. 5 shows the elution profile of the chro-

matography. The main peak (fraction B-1) was eluted in fractions 25 to 29, which eluted with approximately 0.5 M NaCl in a linear gradient. The active components were not retained in cation-exchange chromatography using an S-Sepharose fast flow column (data not shown).

Fraction B-1 thus obtained was then desalted and concentrated on a Sephadex G-50 column. The active fractions were pooled, lyophilized, and further purified by reverse phase HPLC.

Reverse Phase HPLC

The desalted fraction B-1 was purified by reverse

Fig. 4 Re-chromatography on gel filtration of fraction A-3. The conditions of gel filtration were the same as described in Fig. 1. ●—●, inhibitory activity; ○—○, absorbance

Fig. 5 Ion exchange chromatography of fraction A-3. Fraction A-3 in Fig. 4 was dissolved in 10 mM Tris-HCl (pH 7.4), and applied on a Q-Sepharose fast flow column. The column was equilibrated with the starting buffer (10 mM Tris-HCl, pH 7.4) and eluted with a linear gradient of NaCl from 0 to 1 M in 10 mM Tris-HCl (pH 7.4) for 25 min.

phase HPLC on a YMC D-ODS column (20×250 mm). The major peak (fraction C-1) was eluted at 34 min as shown in Fig. 6, which eluted with 25% acetonitrile and showed strong inhibitory activity for [3 H]CCK-8 binding to CCK-B receptor. Fraction C-1 was re-chromatographed on reverse phase HPLC on a YMC D-ODS column (4.6×250 mm) to give a single sharp peak (fraction D-1) at 17 min, when monitored by the absorbance at 210 nm (Fig. 7). The peak fraction was lyophilized and subjected to the sequence analysis.

Analysis of Amino Acid Sequence

Sequence analysis of fraction D-1 was performed

by an automated protein sequencer. The N-terminal amino acid sequence of fraction D-1 proved to be Ser-Tyr-Arg-Glu-Pro-Asn-Thr-.

DISCUSSION

We have found that the Saiko root extract contains components which bind to CCK-B receptor. This novel finding prompted us to isolate the active components from the Saiko root extract.

It has been well-known that CCK is a brain-gut peptide existing in multiple molecular forms with the carboxy terminal octapeptide (CCK-8) (12, 13). In this study, RRA for CCK-8 was successfully used for monitoring the active components which

Fig. 6 Reverse-phase HPLC of fraction B-1. After desalting, lyophilized fraction B-1 was dissolved in 0.01 N HCl, and applied on a column of YMC-pack D-ODS-5-A (20×250 mm I. D., YMC), which was eluted with a linear gradient of acetonitrile from 15% to 50% in 0.01 N HCl for 35 min at a flow rate of 5 ml/min, and absorbance was monitored at 210 nm. A, inhibitory activity; B, absorbance

Fig. 7 Re-chromatography of C-1. Fraction C-1 was re-chromatographed on a column of YMC-pack D-ODS (4.6×250 mm I. D.). The purified fraction (fraction D-1) was lyophilized and used for sequencing.

bind to CCK-B receptors. Water extract of Saiko root was found to contain components having the highest inhibitory activity against [3 H]CCK-8 binding to CCK-B receptors, suggesting that the active substance may possess a water-soluble property. After gel filtration on Sephadex G-50 column, the active components were further purified on an anion-exchange column (Fig. 5). Finally, the active component was purified by the reverse phase HPLC. The major fraction having a strong binding activity to the CCK-B receptor was a polypeptide. Sequence analysis of the peptide revealed that the N-terminal sequence of the peptide consists of 7 amino acid residues, Ser-Tyr-Arg-Glu-Pro-Asn-Thr-. This is the first report demonstrating that the component isolated from Saiko root expresses a high affinity binding activity to CCK-B receptors. In addition, the present study suggested the existence of more than one component in Saiko root having different binding activity to CCK-B receptors, as shown in Figs. 3 and 6. Isolation and identification of these fractions are underway.

CCK has been known to be not only an important endocrine regulator of the gastrointestinal function but also a putative neurotransmitter in the central nervous system (7, 9, 14). The antagonist of CCK could increase food intake (4), enhance morphine analgesia (3, 17) and reduce anxiety (6). It was suggested that CCK antagonists would be useful for treating different diseases regulated by CCK (10). The present study revealed clearly the presence of components in Saiko root exhibiting a strong inhibitory activity for CCK-8 binding to CCK-B receptors. It is presumable that these components may act as a novel CCK antagonist.

The present findings provide an important approach to search for new biologically active substances, such as CCK antagonists from Chinese herbs. In addition, RRA for peptide hormones proved to be a useful tool for finding novel pharmacologically active substances from the Chinese medicine.

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REFERENCES

1. ABE H., ODASHIMA S. and ARICHI S. (1978) The effects of Saikosaponins on biological membranes. *Planta Med.* **34**, 287–290
2. ABE H., SAKAGUCHI M., YAMADA M., ARICHI S. and ODASHIMA S. (1980) Pharmacological actions of Saikosaponins isolated from *bupleurum falcatum*. *Planta Med.* **40**, 366–372
3. DOURISH C. T., O'NEIL M. F., COUGHLAN J., KITCHENER S. J., HAWLEY D. and IVERSEN S. D. (1990) The selective CCK-B receptor antagonist L-365, 260 enhances morphine analgesia and prevents morphine tolerance in the rat. *Eur. J. Pharmacol.* **176**, 35–44
4. DOURISH C. T., RYCROFT W. and IVERSEN S. D. (1989) Postponement of satiety by blockage of brain cholecystokinin (CCK-B) receptors. *Science* **245**, 1509–1511
5. HIAI S., YOKOYAMA H., NAGASAWA T. and OURA H. (1981) Stimulation of the pituitary-adrenocortical axis by Saikosaponin of *Bupleuri radix*. *Chem. Pharm. Bull.* **29**, 495–499
6. HUGHES J., BODEN P., COSTALL B., DOMENEY A., KELLY E., HORWELL D. C., HUNTER J. C., PINNOCK R. D. and WOODRUFF G. N. (1990) Development of a class of selective cholecystokinin type B receptor antagonists having potent anxiolytic activity. *Proc. Natl. Acad. Sci. USA* **87**, 6728–6732
7. JENSEN R. T., LEMP G. F. and GARDNER J. D. (1982) Interactions of COOH-terminal fragments of cholecystokinin with receptors on dispersed acini from guinea pig pancreas. *J. Biol. Chem.* **257**, 5554–5559
8. KUWAHARA T., TAKAMIYA M., NAGASE H., KUDOH T., NAKANO A., YOSHIZAKI H. and ARISAWA M. (1993) GH₃ cells, an anterior pituitary cell line, express CCK-B receptors. *Peptides* **14**, 647–649
9. LAM Y. K., TONY, BOGEN D., CHANG T. S., FAUST K. A., HENSENS O. D., ZINK D. L., SCHWARTZ C. D., ZITANO L., GARRITY G. M., GAGLIARDI M. M., CURRIE S. A. and WOODRUFF H. B. (1991) Novel and potent gastrin and brain cholecystokinin antagonists from *Streptomyces olivaceus*. *J. Antibiot.* **44**, 613–625
10. LUO W. Q., HOSHINO M., SUZUKI M., IGUCHI K., TAKATSUKA N., MOCHIZUKI T. and YANAIHARA N. (1994) Effects of Daisaikoto and Junchoto (traditional Chinese medicine) on release of some gastrointestinal hormones in rats. In *Kiketsusui* 1994 (ed. YANAIHARA N.) Kiketsusui Conference, Shizuoka, pp 23–28
11. MULLER J. E., STRAUS E. and YALOW R. S. (1977) Cholecystokinin and its COOH-terminal octapeptide in the pig brain. *Proc. Natl. Acad. Sci. USA* **74**, 3035–3037
12. REHFELD J. F. (1978) Immunochemical studies on cholecystokinin. *J. Biol. Chem.* **253**, 4022–4030
13. REHFELD J. F. (1985) Neuronal cholecystokinin: one or multiple transmitters? *J. Neurochem.* **44**, 1–10
14. TAKAMIYA M., YOSHIZAKI H., HANAYAMA A., NAGASE H., KUDOH T., KUWAHARA T. and ARISAWA M. (1991) The presence of cholecystokinin B receptors in rat pituitary gland and GH₃, a rat anterior pituitary tumor cell line. *Jap. J. Pharmacol.* **55**, Suppl. 1, 355
15. TAKEDA Y., HOSHINO M., YANAIHARA N., YANAIHARA C., ISOBE J., SUGIURA N., KASHIMOTO K., TAKANO Y. and KAMIYA H. (1989) Comparison of CCK-8 receptors in the pancreas and brain of rats using CCK-8 analogs. *Jap. J. Pharmacol.* **49**, 471–481
16. WIESENFELD-HALLIN Z., XU X.-J., HUGHES J., HORWELL D. C. and HOKFELT T. (1990) PD134308, a selective antagonist of cholecystokinin type B receptor, enhances the analgesic effect of morphine and synergistically interacts with intrathecal galanin to depress spinal nociceptive reflexes. *Proc. Natl. Acad. Sci. USA* **87**, 7105–7109
17. WILLIAMS J. A. (1982) Cholecystokinin: A hormone and a neurotransmitter. *Biomedical Res.* **3**, 107–121