Growth of Breast Cancer Cells Inhibited by Bromelains Extracted from the Different Tissues of Pineapple

Shu HAIYAN, Ma FUNING, Li KEMING, Sun WEI, Xu GUIYING, Zhan RULIN, and Chang SHENGHE

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Original article


Breast cancer is a common disease diagnosed in women. The therapies for breast cancer often have side effects and the most effective drugs are highly toxic. Finding safe alternative medicines is an important goal in the search for a cure for breast cancer. Bromelains are a mixture of cysteine proteases extracted from pineapple. Pineapple is considered to be a safe food. In this paper, the breast cancer cell lines MCF7 and MDA-MB-231 were co-cultured with bromelains extracted from different tissues of the pineapple. Results showed that the growth of MCF7 and MDA-MB-231 cells can be inhibited by bromelains. The bromelains for this study were extracted from the fruit pulp, peel, fruit stalk, young stem, and mature stem. Bromelains extracted from the fruit stalk were found to be the most effective for inhibiting the growth of breast cancer cells. MCF7 was more sensitive to bromelains than MDA-MB-231. After the breast cancer cells were co-cultured with the bromelain, the transcripts of p53 and Bax increased significantly. The transcriptional expressions of Cox-2 and Bcl-2 were down-regulated. More proteins of p53 and Bax were produced. The translational expressions of Cox-2 and Bcl-2 were both down-regulated. Bromelains inhibited the growth of BC cells through the up-regulating of p53, Bax, and by decreasing the expressions of Cox-2 and Bcl-2. The activity of nuclear factor-kappa B (NF-xB) might be blocked. Final effects might be performed by regulating the functions of the protein kinase.

Key words: Bromelains, breast cancer, fruit stalk, p53, inhibition.

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Breast cancer is the most common type of cancer diagnosed in women (MOHAMAD et al. 2019; KHAZAYEL et al. 2017). The incidence rate of breast cancer is higher in developed countries than in developing countries (BECKER 2010; FERALY et al. 2010). Breast cancer is a major cause of death from cancer in women (ZARE et al. 2015; BRAY et al. 2018). More than 600,000 women die from breast cancer every year and more than one million new cases of breast cancer are diagnosed every year (RAGHAVENDRA et al. 2017; SHAHZAMANI et al. 2016). Because of the heterogenic nature of malignant tumors, the crosstalk between different cell signaling systems, and the development of drug resistance, breast cancer is a heavy burden for many families all over the world (GONZALEZ-ANGULO et al. 2007; COLLEONI et al. 2009; ARPINO et al. 2009). These days, the treatment of breast cancer can include surgery, chemotherapy, radiotherapy, and so on (MILLER et al. 2017; FERNANDES et al. 2018). Patients who receive such treatments always have many side effects. For example, well-known breast cancer drugs such as docetaxel, tamoxifen, and cisplatin (Early Breast Cancer Trialists’ Collaborative, 2012) are always likely to induce inflammation, which can promote tumor progression and metastasis (VYAS et al. 2014; JIN et al. 2015). Furthermore, many drugs themselves are highly toxic to normal cells (HOLOHAN et al. 2013; GARRAWAY & JANNE 2012). The need for a safe medicine for treating breast cancer is urgent. Searching a safe treatment or medicine for breast cancer is the main direction of many scientists and doctors’ research.

Bromelain is a bioactive agent extracted from pineapples (Ananas comosus). It is sold as a dietary supplement and considered to be a safe food (HALE 2004), so it can be assumed that bromelain has no
Bromelain extraction

The pineapple fruit pulp, peel, stalk, and stem were cut and crushed in a juice extractor. The mixture was filtered through gauze. The crude filtrate was centrifugated at 8000 g, 10 min, 4°C. The supernatant was collected and put in a 1-liter breaker. A forty percent ammonium sulfate solution was added and mixed until all of the ammonium sulfate was dissolved. The mixture was dialyzed overnight. The dialysate was replaced with fresh pure water and dialyzed overnight once again. 0.1 mol/l BaCl₂ solution was added into the dialysate. The dialysis experiment continued until there was no precipitate in dialysate when 0.1 mol/l BaCl₂ solution was added. The solution in the bag filter was collected for experimentation. For a blank control, all procedures were performed as described above except that the crude filtrate from pineapple tissues was replaced with 40% ammonium sulfate solution.

Measurement of bromelain activity

The concentration of bromelains was measured using a Bradford Protein Assay Kit (Beyotime Biotechnology, Shanghai, China) according to protocol. Enzyme activity was measured according to the method described by Lauwers (1975) and modified. Five milliliters of tyrosine solution was put in a 15 ml test tube. The test tube was put into a 37°C water-bath for 10 minutes. A specific bromelain was diluted with L-cys and EDTA-Na₂ and added into the tyrosine solution described above. The mixture was then shaken and put into a 37°C water-bath for 10 minutes. Then, 5 ml of trichloroacetic acid (TCA) was added to the mixture to stop the reaction. The test tube was shaken and put into a 37°C water-bath for 30 minutes. The test tube was then cooled to room temperature. Next, the mixture was centrifugated at 3500 rpm for 10 minutes at 20°C. The supernatant was collected and the light absorption of value A was measured at 275 nm. Another equal portion of enzyme solution was treated. The procedure of adding the enzyme solution and that of adding TCA was repeated. The light absorption value was noted as A₀. The enzyme activity was calculated as the following:

\[ E = \frac{A - A_0}{A_w \times M \times t \times \text{diluted times}} \]

Materials and Methods

Materials

Pineapple (Ananas comosus var. ‘Comte de Paris’) suckers were planted in an experimental field of Haikou Station, Chinese Academy of Tropical Agricultural Sciences, on August 10th, 2017. Flower forcing was performed on August 10th, 2018. The resulting fruits were harvested on January 10th, 2019. Young stems were collected on August 10th, 2018. Fruit pulp, peel, stalk, and the mature stem were collected on January 10th, 2019.

Measurement for survival rate of tumor cells

Breast tumor cell lines MCF7 cells and MDA-MB-231 cells (Center of Shanghai Cell Resource, Chinese Academy of Sciences) were seeded into each well of
a 96-well microculture plate (TPP, Switzerland) at a concentration of 2 × 10^5 cells/ml. Simultaneously, twenty or forty microliters of bromelain solution from different tissues were added to 1 ml of complete L-15 medium. The final concentration was 15 µg/ml and 30 µg/ml, respectively. One milliliter of the mixture was added into each of the 96-well microculture plates containing cells. After 72 h of incubation (incubation time based on ECKERT et al. 1999), cell viability was assessed by a MTT assay (Promega, USA) according to the manufacturer’s instructions. For the control experiment, all of the procedures were performed as described above except that the twenty microliter bromelain solution was replaced with twenty microliters of the blank solution described in the part “measurement of bromelain activity”. All the experiments were performed in three biological replicates and each with triplicates.

Quantitative real time-polymerase chain reaction (qRT-PCR)

MCF7 cells (6×10^5) were seeded in complete L-15 medium in 6-well plates and incubated over 24 h. Then, the medium was renewed with L-15 in the presence of bromelains (0 µg/ml, 15 µg/ml, 30 µg/ml). After incubation for 72 h, total RNA was extracted with a Trizol reagent (Invitrogen, American) according to the manufacturer’s protocol and 4 µg of RNA was converted to cDNA using a PrimeScript™ RT reagent kit (TaKaRa, Dalian, China). The housekeeping gene, β-actin served as the internal control. Each real-time PCR reaction contained 10 µl of SYBRR mix, 1 µl of each Forward/Reverse Primer (400 nM), 1 µl of cDNA solution, 0.4 µl of Dye, and with the addition of dH2O, the final volume was 20 µl. PCR cycles consisted of: 2 minutes at 50°C, 4 minutes at 95°C for polymerase activation, 45 cycles of 10 seconds at 95°C (denaturation), 5 seconds at 54°C, 5 seconds at 72°C, and 15 seconds at 83°C (annealing and extension). Finally, melting was carried out at 72-95°C (in 0.5°C increments) for 5 seconds for each step. All PCR reactions were performed in triplicate using the mean value being used to determine mRNA levels. Relative mRNA expression levels for each gene were analyzed using the 2-ΔΔCt method and normalized to the endogenous reference gene β-actin. The primers used in this research were shown in Table 1.

Western blotting analysis

In this study, Western blotting was used to determine the expression of p53, Bax, Cox-2, Bcl-2 and Actin. β-actin 42 kDa was selected as the reference protein. Antibodies used for western blot analysis include p53, Bax, Cox-2, Bcl-2, and β-actin and were purchased from Sigma-Aldrich (Beijing, China). The antibody-antigen complexes were subsequently identified using horseradish peroxidise (HRP). The blot was visualized using chemiluminescent detection substrate. A chemiluminescence ONE-HOUR Western™ Advanced Kit (Genscript Company, Beijing, China) was used in these experiments. Quantity One4.6.2 (Bio-Rad) software was used to quantify the band intensity for all the targeted proteins. The relative expression analyzed of p53, Bax, Cox-2, Bcl-2, and Actin between the control and the bromelain treated cells was calculated using Plot Density (Bio-Rad).

Table 1

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin(F)</td>
<td>5’-TCT GCC ACC ACA CCT TCT ACA ATG-3’</td>
<td>PORICHI et al., 2009</td>
</tr>
<tr>
<td>β-actin(R)</td>
<td>5’-AGC ACA GCC TGG ATA GCA ACG-3’</td>
<td>PORICHI et al., 2009</td>
</tr>
<tr>
<td>Bcl-2 (F)</td>
<td>5’-ATCGCCCTGTGGATGACTGAG-3’</td>
<td>PORICHI et al., 2009</td>
</tr>
<tr>
<td>Bcl-2 (R)</td>
<td>5’-CAGCCAGGAGAAATCAAACAGAGG-3’</td>
<td>PORICHI et al., 2009</td>
</tr>
<tr>
<td>Bax (F)</td>
<td>5’-GGAACGAACTGGACAGTAACATGG-3’</td>
<td>PORICHI et al., 2009</td>
</tr>
<tr>
<td>Bax (R)</td>
<td>5’-GCAAAGTAGAAAAGGGCGACAAC-3’</td>
<td>PORICHI et al., 2009</td>
</tr>
<tr>
<td>P53(F)</td>
<td>5’-ATTTGCGTGTGGAGTATTTG-3’</td>
<td>STARK et al., 2006</td>
</tr>
<tr>
<td>P53(R)</td>
<td>5’-GGACAAGAAGTAGAAAGGGCGACAAC-3’</td>
<td>STARK et al., 2006</td>
</tr>
<tr>
<td>Cox-2(F)</td>
<td>5’-AAGTCCTGTGACCAGATCTACG-3’</td>
<td>Li et al., 2012</td>
</tr>
<tr>
<td>Cox-2(R)</td>
<td>5’-TTCTACCACCAGCAACC-3’</td>
<td>Li et al., 2012</td>
</tr>
</tbody>
</table>
Statistical analysis

In each experiment, 40 samples were used. All of the data fulfil Anova assumptions. Statistical tests were performed using Anova with post-hocs. The results were expressed as mean ± SD. Statistical analysis was done using SPSS version 17.0 (SPSS Inc., Chicago, USA). Probability values of less than alpha 0.05 (p < 0.05) were considered statistically significant.

Results

The growth of breast cancer cells can be inhibited by bromelains

The difference of the activity of enzymes from different tissues was significant. Bromelain from fruit pulp had the most activity. The lowest bromelain activity was from the fruit peel. The sequence of bromelain activity from most to least was pulp (728±51 U/g) > young stem (534±39 U/g) > fruit stalk (479±28 U/g) > mature stem (290±20 U/g) > peel (271±18 U/g) (Fig. 1). This indicated that pineapple pulp was the best tissue for extracting bromelain. The bromelains (15 µg/ml) from different tissues were added into breast tumor cell lines and cultured respectively. After 72 hours, the survival rate of the cells was measured. Results showed that for cell line MCF7, the survival rate of the cells co-cultured with bromelain from the fruit stalk was the lowest compared to the other sources of bromelain (Fig. 2). The cells co-cultured with bromelain extracted from the fruit peel had the highest survival rate. The sequence of the survival rates of the cell line co-cultured with bromelains extracted from different tissues was pulp > peel > young stem > mature stem > fruit stalk (Fig. 2). For cell line MDA-MB-231, the sequence of the survival rates of the cell line co-cultured with bromelains extracted from different tissues was pulp > peel > mature stem > young stem > fruit stalk (Fig. 2). When 30 µg/ml of bromelains from different tissues was added to the breast tumor cell lines, the survival rate of the cells was significantly lower than those treated with 15 µg/ml of corresponding bromelains (data not shown). But the sequence of the survival rate of cell lines co-cultured with bromelains from different tissues was the same with that of cell lines treated with 15 µg/ml. This demonstrated that bromelains have a significant inhibitive effect on the growth of breast tumor cells. Bromelain extracted from the pineapple fruit stalk is the most inhibitive in the growth of breast cancer cells. Enzyme activity did not correspond with the inhibiting effects. For each group of bromelain from the same tissue, the survival rate of MDA-MB-231 was higher than that of MCF7. For MDA-MB-231 the survival rate of the cells co-cultured with bromelain from the fruit peel was the highest. For MCF7, the cells co-cultured with bromelain from the fruit peel had the highest survival rate (Fig. 2). This demonstrated that different cell lines might have different sensitivities to bromelain.

The transcriptional expression of signal genes after being treated with bromelain

In order to study the mechanism that allowed bromelain to inhibit the growth of breast tumor cell lines, the transcriptional expressions of p53, Bax, Cox-2, and Bcl-2 in MCF7 co-cultured with 15 µg/ml of bromelain extracted from different tissues were researched. Results showed that after MCF7 cells were co-cultured with bromelain (15 µg/ml) for 72 hours, the mRNAs of p53 were significantly higher than that of the control (Fig. 3B). The transcriptional expression of p53 was induced after MCF7 cells were co-cultured with bromelains. Among the five treatments, the transcription level of p53 in MCF7 cells co-cultured with bromelain from the fruit stalk was the highest. The transcription level of the data from the fruit pulp was the lowest. The sequence of the transcription level of p53 in MCF7 cells co-cultured with bromelain from the fruit stalk was the highest. The transcription level of the data from the fruit stalk was the lowest. The sequence of the transcription level of p53 in MCF7 cells co-cultured with bromelain from tissues was fruit stalk > mature stem > young stem > peel > pulp (Fig. 3B). The transcription level of Bax was also induced (Fig. 3C). However, the
transcription levels of Cox-2 and Bcl-2 were both down-regulated. For example, in MCF7 cells, the relative expression of Cox-2 cells without bromelain was 10.58±0.05 (Fig. 3D). The corresponding data of cells co-cultured with 15 µg/ml of bromelain from the fruit stalk for 72 hours was 9.02±0.04 (Fig. 3D). The relative expression of Cox-2 in the cells co-cultured with bromelains was significantly lower than that of the control (Fig. 3D). After being co-cultured with bromelains from different tissues, the sequence of transcription levels of Cox-2 was pulp > peel > young stem > mature stem > fruit stalk (Fig. 3D). Similar results were recorded for the expression of Bcl-2 (Fig. 3E). This demonstrated that after MCF7 cells were co-cultured with bromelains, the transcriptions of p53 and BAX were induced while the transcriptions of Cox-2 and Bcl-2 were down-regulated.

The translational levels of p53 and Bax were up-regulated by bromelains

Protein was the final executor for gene expression. A high level of transcriptions does not always mean more protein. Therefore, the protein levels of signal genes after the MCF7 cells were co-cultured with bromelain were studied. Results showed that after breast tumor cells were cultured with 15 µg/ml of bromelain extracted from the fruit stalk, the average quantity of the western-blotting band for p53 was 190.73±8 intensity (int) (Fig. 4A and 4B). The corresponding data of the protein level for p53 when 30 µg/ml of bromelain extracted from fruit stalk was co-cultured was 236.72±9 int (Fig. 4A and 4B). The corresponding data of the control was only 149.52±9 int (Fig. 4A and 4B). The protein level of p53 increased significantly compared to the control. The protein level of p53 in MCF7 cells co-cultured with 30 µg/ml bromelain for 72 hours was higher than that of p53 in MCF7 cells co-cultured with 15 µg/ml of bromelain for 72 hours (Fig. 4A and 4B). This demonstrated that the protein level of p53 was up-regulated by bromelain. Similar effects can also be found in the protein expression of Bax (Fig. 4C and 4D). When MCF7 cells were cultured without bromelain, only a faint band corresponding to Bax could be found in the film (Fig. 4C). When bromelain was added into the cell cultures, clearer bands were found at the same molecular-weight positions (Fig. 4C). In contrast to the protein expression of p53 and Bax, the protein level of Cox-2 decreased remarkably after bromelain was added into the cell cultures (Fig. 4G and 4H). The average quantity of the protein level for Cox-2 when treated with 30 µg/ml of bromelain was less than those treated with 15 µg/ml of bromelain and the control (Fig. 4G and 4H). Similar results were found for the protein expressions of Bcl-2 (Fig. 4E and 4F). This showed that the protein expressions of Cox-2 and Bcl-2 were both regulated negatively by bromelain.
Discussion

Bromelain is a mixture of many cysteine proteases. It can digest dead cells and proteins. Bromelain can alleviate the symptoms of inflammation. Many cancers are caused by long-term inflammation (BHUI et al. 2009). Some papers suggested that since bromelain can alleviate the symptoms of inflammation, it could possibly inhibit the growth of tumor cells. In this study, we found that after breast cancer tumor cells were co-cultured with bromelains for 72 hours, the growth of tumor cells was inhibited. This demonstrated that bromelains can restrain the growth of tumor cells. Bromelains can be used clinically for alleviating the pain of breast cancer patients.

Fig. 4. Western blotting for signal genes after breast cell lines were co-cultured with bromelain extracted from the fruit stalk. Data are expressed as mean ± SD of triplicates; statistically significant differences at *p < 0.05.
tracted from the pineapple fruit pulp, peel, stalk, young stem, and mature stem, the survival rate of those co-cultured with bromelains extracted from the fruit stalk was the lowest. For inhibiting the growth of BC tumor cells, bromelains from fruit stalk were the most effective. For each treatment with bromelains extracted from different tissues, the survival rate of MCF7 was lower than that of MDA-MB-231. This demonstrated that MDA-MB-231 cells were less sensitive to bromelains than MCF7. The effects of bromelains on MCF7 was better than that on MDA-MB-231.

Although bromelains were found to inhibit the growth of tumor cells, the underlying mechanisms remained unclear. In vitro, bromelain inhibited tumor cell proliferation, invasion, and expression of CD44 surface molecules (GRABOWSKA et al. 1997; HARRACH et al. 1994; KLEE et al. 1996). Another mechanism may involve the induction of tumor cell differentiation, as demonstrated with leukemic cells (MAURER et al. 1988). ECKERT et al. (1999) reported that the benefits of bromelains for BC patients was, that they could stimulate the suppressed immunotoxicity of monocytes in BC patients. Bax, p53, Cox-2, and Bcl-2 are major culprits for cancers. Published papers reported that these molecules were regulated when cancer patients were cured (SARKAR & Li 2004). After MCF7 cells were co-cultured with bromelains, the transcriptional expressions of p53 and Bax were both up-regulated. The transcripts of Cox-2 and Bcl-2 both decreased significantly. On the protein level, after BC cells were co-cultured with bromelains, many more proteins of p53 and Bax were produced, while the quantities of Cox-2 and Bcl-2 were reduced remarkably. This showed that the inhibitive effects of bromelains on the growth of BC cells was through the up-regulating of p53, Bax, and by the decreasing of the expressions of Cox-2 and Bcl-2. The activity of nuclear factor-kappa B (NF-κB) might be blocked. Final effects might be performed by regulating the functions of the protein kinase (BHUI et al. 2009).

Conclusions

We reported that the growth of the breast cancer tumor cell lines MCF7 and MDA-MB-231 can be inhibited by bromelains. For inhibiting the growth of BC tumor cells, bromelains from the pineapple fruit stalk was more effective than those from other tissues. MDA-MB-231 cells were less sensitive to bromelains than MCF7 cells. The inhibitive effects of bromelains on the growth of BC cells was through the up-regulating of p53, Bax, and by the decreasing of the expressions of Cox-2 and Bcl-2. The activity of nuclear factor-kappa B (NF-κB) might be blocked. Final effects might be performed by regulating the functions of the protein kinase.

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Author Contributions

Research concept and design: S.H., S.C.; Collection and/or assembly of data: S.H., F.M., S.C.; Data analysis and interpretation: S.H., S.C., K.L.W.S., G.X.; Writing the article: S.H.; Critical revision of the article: S.C., R.Z.; Final approval of article: S.H.

Conflict of Interest

The authors declare no conflict of interest.

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