**In Vivo** Antitumor and Antioxidative Effects of a Rapeseed Meal Protein Hydrolysate on an S180 Tumor-Bearing Murine Model

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The antitumor and antioxidative activities of a rapeseed protein hydrolysate (RSCH) obtained from rapeseed meal were evaluated by using an in vivo S180 tumor-bearing Kunming mouse model. Tumor-bearing female mice were given RSCH for 10 at doses of 0, 50, 100, and 150 mg/kg/d by gastric perfusion. RSCH significantly decreased the tumor weight by 44% and 53% in the 100 and 150 mg/kg/d groups, respectively, without causing mortality or growth retardation. The thymus and spleen indices (organ weight relative to body weight) were increased significantly in the 150 mg/kg/d group. The phagocytic capability of coeliac macrophages and delayed-type hypersensitivity (DTH) were significantly increased in tumor-bearing mice treated with RSCH at 150 mg/kg/d. RSCH administration also enhanced the superoxide dismutase activity and reduced the serum level of thiobarbituric acid reactive substances. Our results show that an oral RSCH administration had an antitumor protective effect and may improve immune function by reducing free radical formation and oxidative stress in a murine model.

**Key words:** rapeseed protein hydrolysate; antitumor; antioxidant; tumor-bearing mice

Bioactive peptides have been defined as specific protein fragments having a positive impact on human health.1,2 The beneficial effects of bioactive peptides have been classified as antimicrobial, antioxidative, antithrombotic, antihypertensive, or immunomodulatory.2,3 Cancer is a disease manifested by uncontrolled cell growth, and the development of an effective tumor-inhibiting agent for cancer represents a unique scientific challenge. In recent years, there has been a growing interest in the identification and characterization of natural antitumor and antioxidative agents in human food. Food rich in antioxidants such as ascorbic acid, tocopherols, polyphenols, and flavonoids, which prevent free radical damage,4 has been found to be inversely associated with the risk of cancer and other chronic diseases.5 Peptides and proteins from food sources have also aided cancer prevention and treatment. For example, whey proteins and α-lactalbumin have been shown to inhibit colonic cell proliferation.6 Soy products have been associated with a decreased risk of prostate,7–9 breast,10 and endometrial cancer.11

Rapeseed is the world’s second leading source of protein meal. Defatted rapeseed meal contains about 32% protein12 which could potentially be used as a food ingredient instead of being wasted or used as animal feed. Since rapeseed constitutes an interesting raw material for the preparation of a protein isolate and hydrolysate, researchers have tried to utilize a rapeseed protein isolate and its defatted meal as a source of bioactive peptides to enhance the value of the rapeseed hydrolysates. For example, Marczak and others have used subtilisin to produce antihypertensive peptides from rapeseed protein.13 Yust and others have reported the HIV protease inhibitory activity of a rapeseed protein hydrolysate which produced by using Alcalase.14 Very recently, we have reported a rapeseed protein hydrolysate (RSCH) and its three peptide fractions (RSP1, RSP2, and RSP3) separated by Sephadex gel filtration, exhibited dose-dependent antioxidative power and inhibited superoxide anion generation and malondialdehyde (MDA) formation in a rat liver tissue homogenate.15 These results suggested that the rapeseed protein hydrolysate could be useful as a human food additive for a source of bioactive peptides with antioxidative properties. Nevertheless, whether rapeseed protein has antitumor and antioxidative potentials, both in vitro and in vivo, has not been investigated. Considering the above-mentioned immunopotentiating activities of the rapeseed peptide fractions, it would be of interest and importance to examine whether the rapeseed protein hydrolysate possesses antitumor and antioxidative effects in vivo by using an animal model.

The present study reports on the in vivo antitumor and antioxidative activities of the rapeseed protein hydrolysate obtained from rapeseed of the Huaza3 variety which is widely planted in China due to its high oil and low erucic acid and glucosinolate content. Our work is aimed at investigating the in vivo inhibiting efficacy of hydrolyzed rapeseed peptides on tumor-bearing murine animals, thus evaluating its use for human food nutrition as a source of bioactive peptides with antitumor and antioxidative properties.

**Materials and Methods**

Preparation of the rapeseed peptide hydrolysate. The Rapeseed peptide crude hydrolysate (RSCH) was prepared as previously...
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In brief, defatted and dehulled meal of rapeseed variety Huaa3 was ground in a 5% NaCl (w/v) solution and then centrifuged at 4,000 g for 20 min. The pellet was dissolved in distilled water and centrifuged again to obtain the albumin fraction. The Albumin isolate (5% w/v) was then hydrolyzed by sequential treatment with Alcalase (0.2 AU/g of substrate, 1 h) and Flavourzyme (50 LAPU/g of substrate, 2 h) at 50°C and pH 8. After the hydrolysis had been stopped by heating at 80°C for 10 min, the hydrolysate was clarified by centrifugation at 4,000 g for 10 min, the supernatant being designated as the rapeseed protein crude hydrolysate (RSCH) for use in further analyses.

Animals and experimental conditions. Female Kunming nude mice (18–22 g), aged 5–6 weeks, were obtained from the Animal Center, Institute of Health and Epidemic Prevention (Wuhan, China). All animal procedures were approved by the Animal Care and Use Committee of the Institute of Health and Epidemic Prevention (Wuhan, China). The mice, which were housed in accordance with institutional animal care policies, had access to water and standard laboratory feed. The Animals were randomly divided into five groups: control and four tumor-bearing groups treated with 0, 50, 100 and 150 mg/kg/d of RSCH, respectively.

Tumor implantation. Murine sarcoma S180 cells were provided by Tongji Medical School, Huazhong Science and Technology University (Wuhan, China) and cultured in an RPMI-1640 medium supplemented with 10% heat-inactivated calf serum, 100 U/ml of penicillin G, and 100 U/ml of streptomycin at pH 7.4 in a humidified CO2 incubator (Sanyo, Japan) with a 5% CO2 atmosphere at 37°C.

Tumor-bearing mice prepared by transplanting sarcoma 180 cells (S180) were used in this work due to their practicality as a consistent and short preparation time animal model. The S180 cells were harvested and washed with PBS and adjusted to a concentration of 1 × 107 cells/ml. The mice were implanted subcutaneously on the midrigh side with 0.2 ml of the S180 cells in PBS. The day of inoculation is defined as day 0.

Tumor weight and relative thymus and spleen weights. On the tenth day of oral administration, the mice were weighed and sacrificed by cervical dislocation. The tumor, spleen and thymus were excised from each animal and immediately weighed. The thymus and spleen indices are expressed as the organ weight relative to body weight.

Macrophage phagocytosis assay. The phagocytic capacity of peritoneal cavity phagocytes was assessed by following the method previously described. On the tenth day of the experiment, the mice were injected intraperitoneally with 1.0 ml of 1% chick red blood cells (CRBC). After 30 min, the mice were sacrificed and intraperitoneally injected with a Hanks solution, and the lavage fluid was obtained for a macrophage phagocytosis assay. The cells were smeared on a slide, dried, stained with Wright’s stain for 0.5–1 min and then washed with a phosphate buffer. The cells were microscopically counted under oil immersion. The phagocytic index is expressed in two ways: (i) the number of CRBC ingested by 100 phagocytes is expressed as the “phagocytosis index”; (ii) percentage of phagocytes that phagocytosed the red blood cells is expressed as the “phagocytic percentage.”

Induction of contact sensitivity with DNF. On the fifth day of the RSCH oral administration, 25 μl of 1% dinitrofluorobenzene (DNFB) in 4:1 acetone:olive oil was painted on the shaved mouse abdomen on consecutive days (days 0 and 1). Five days later (day 5), 10 μl of DNFB in the same vehicle was applied to the dorsum of the right ear. The mice in the control groups were only challenged by the vehicle without the DNF agent. The mice were sacrificed by cervical dislocation after 24 h, and ear tissue of 8 mm in diameter was punched from two ears. The contact sensitivities to DTH were quantified by determining the weight increase in the right ear relative to the left ear.

Organ histopathological evaluation. The organs/tissues were fixed in 10% (v/v) neutral formalin, embedded in paraffin, and sectioned in 5-μm-thick slices. The sections were stained with hematoxylin and eosin (HE) stain for subsequent microscopic examination.

Results and Discussion

Effect of RSCH on the growth of tumors implanted in mice

There was noticeable growth of S180-induced tumors in the subcutaneous tissues due to the proliferation of tumor cells (Table 1). Ten days after the implantation, the tumor weight in the mice not treated with RSCH was found to be 1.48 ± 0.19 g (Table 1). The average tumor weight in the three RSCH treatment groups was about 66% (50 mg/kg/d), 56% (100 mg/kg/d) and 47% (150 mg/kg/d), compared to that of the untreated mice, the inhibitory effect becoming more evident with increasing administration dose of RSCH.

The results of the tissue examination of the thymus and spleen are shown in Table 2. Compared to the normal nude control, all tumor-bearing groups exhibited a lower thymus index and higher spleen index (Table 2).

Table 1. Antitumor Activity of the Rapeseed Protein Crude Hydrolysate (RSCH) in Mice with Transplanted S180 Tumors

<table>
<thead>
<tr>
<th>Group</th>
<th>Tumor weight (g)</th>
<th>Tumor Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nude control</td>
<td>6.15 ± 0.39</td>
<td>—</td>
</tr>
<tr>
<td>Tumor-bearing RSCH 0</td>
<td>6.28 ± 0.19</td>
<td>1.48 ± 0.19</td>
</tr>
<tr>
<td>Tumor-bearing RSCH 50</td>
<td>6.36 ± 0.30</td>
<td>0.97 ± 0.21</td>
</tr>
<tr>
<td>Tumor-bearing RSCH 100</td>
<td>6.61 ± 0.43</td>
<td>0.83 ± 0.23</td>
</tr>
<tr>
<td>Tumor-bearing RSCH 150</td>
<td>6.82 ± 0.19</td>
<td>0.69 ± 0.15</td>
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</tbody>
</table>

Table 2. Effect of the Rapeseed Protein Crude Hydrolysate (RSCH) on Immune Organs in the Tumor-Bearing Mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Thymus index (mg/g)</th>
<th>Spleen index (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nude control</td>
<td>5.40 ± 0.56</td>
<td>3.03 ± 0.22</td>
</tr>
<tr>
<td>Tumor-bearing RSCH 0</td>
<td>2.02 ± 0.65</td>
<td>6.43 ± 0.42</td>
</tr>
<tr>
<td>Tumor-bearing RSCH 50</td>
<td>3.82 ± 1.27</td>
<td>6.64 ± 0.94</td>
</tr>
<tr>
<td>Tumor-bearing RSCH 100</td>
<td>3.91 ± 0.53</td>
<td>6.64 ± 0.67</td>
</tr>
<tr>
<td>Tumor-bearing RSCH 150</td>
<td>3.73 ± 1.00</td>
<td>7.82 ± 0.55</td>
</tr>
</tbody>
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Each value is the mean ± SD of eight independent determinations. Letters indicate significant differences: a different from the tumor-bearing control group, p < 0.05; b different from the tumor-bearing control group, p < 0.01.
nonspecific host-defense mechanisms.18,19) Our results indicate that RSCH may be an activator of cell-mediated immunological memory. 20) In the present study, compared to normal mice, the antigen-specific delayed-type hypersensitivity response was shown to be considerably suppressed in the tumor–bearing mice which signaled the failure of one or more components of the host-defense system. Upon the oral supplementation with RSCH, the induction of DTH response was enhanced in the 150 mg/kg/d dose group (Fig. 2) which represented an increased resistance to infection and an improved host defense capability. This demonstrates that RSCH had the potential to recruit antigen-specific T cells and modulate the T cell-mediated memory response, and thus be useful as an effective nutrient supplement with immune functions.

**Antioxidative effect of RSCH in mice serum**

At a high dose, the administration of RSCH induced a significant rise (14%) in serum SOD activity (Fig. 3). This indicates that the antioxidative activity of RSCH could contribute to its anticarcinogenic activity. Superoxide (O$_2^-$) is believed to be the cause of other ROS formation and the O$_2^-$ scavenging capacity of the body is the first line of defense against oxidative stress. It has been reported that the overexpression of superoxide dismutase and catalase in transgenic flies extended the life-span by as much as one third, perhaps due to decreased oxidative stress.21)

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### Table 3. Effect of the Rapeseed Protein Crude Hydrolysate (RSCH) Administration on Macrophage Infection by CRBC in Mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Phagocytic percentage (%)</th>
<th>Phagocytosis index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nude control</td>
<td>6.25 ± 0.06</td>
<td>7.25 ± 0.21</td>
</tr>
<tr>
<td>Tumor-bearing RSCH 0</td>
<td>3.75 ± 0.06</td>
<td>5.25 ± 0.06</td>
</tr>
<tr>
<td>Tumor-bearing RSCH 50</td>
<td>4.56 ± 0.27</td>
<td>5.75 ± 0.08</td>
</tr>
<tr>
<td>Tumor-bearing RSCH 100</td>
<td>4.81 ± 0.17</td>
<td>6.16 ± 0.15</td>
</tr>
<tr>
<td>Tumor-bearing RSCH 150</td>
<td>5.62 ± 0.22$^a$</td>
<td>7.08 ± 0.41$^a$</td>
</tr>
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Each value are the mean ± SD of eight independent determinations. Letters indicate significant difference: $^a$different from the tumor-bearing control group, $p < 0.05$.

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**Fig. 1.** Histopathological Findings in Tumors Stained with Hematoxylin and Eosin (HE).

A, HE-stained section of tumors in the untreated group (100×); B, HE-stained section of tumors in the 100 mg/kg/d RSCH-treated group (100×).

Compared to the non-treated tumor-bearing group, all three RSCH-treated groups showed an increased thymus index, although only the medium- and high-dose groups reached a significant difference. In respect spleen index compared with the non-treated tumor-bearing group, only the high-dose group exhibited any significant difference (Table 2). No gross abnormalities of toxicological significance were noted for any animal during the necropsy examination (data not shown).

The tumor tissue histopathology is presented in Fig. 1. At the termination of the study, the mice’s neoplasm in the untreated and three RSCH-treated groups was examined by hematoxylin and eosin (HE) staining. There was significant difference between non-treated group and 100 and 150 mg/kg/d RSCH treated groups. In the 0 mg/kg/d RSCH treated group (Fig. 1A), the neoplastic cells show great diversity in size. They were densely stained and grouped together with enlarged nuclei. Greater inconsistency of nuclear membranes and disappearance of the cell organ structure were apparent. Treatment with 100 and 150 mg/kg/d of RSCH resulted in a marked morphological change, the size of nuclei and nucleus lission being significantly less (Fig. 1B). Since that RSCH-treated liver of the tumor-bearing mice exhibited only mild dysplasia and did not display any advanced nuclear atypia commonly seen in the liver of the control tumor-bearing mice, RSCH could have normalized the mouse liver histology and reduced liver pathology.

**Macrophage phagocytosis activity**

Among the three RSCH administration groups, only the high-dose group was found to be able to significantly increase the macrophage activity (Table 3). There was also an increase in the number of CRBC engulfed per macrophage at the 150 mg/kg/d dose. Phagocytosis of pathogens by macrophages initiates the innate immune response, which in turn orchestrates the adaptive response; thus it is one of the most important nonspecific host-defense mechanisms.18,19) Our results support the notion that RSCH may be an activator of macrophage phagocytosis at higher concentrations under the tumor-bearing situation, and a stimulant; RSCH could possess the ability to induce macrophage activity in vivo.

**Fig. 2.** Effect of Administering the Rapeseed Protein Crude Hydrolysate (RSCH) on the Delayed-Type Hypersensitivity (DTH) Reaction in the Murine Model.

Each value is the mean ± SD of ten independent determinations. Letters indicate significant difference: $^a$different from the tumor-bearing control group, $p < 0.05$.

**Delayed type of hypersensitivity (DTH)**

DTH is a reaction triggered by antigen-specific T cells that can be induced by different allergens. Since DTH has been shown to be dependent on specific T memory cells, it is widely used as an in vivo measure of the T cell-mediated immunological memory.20) In the present study, compared to normal mice, the antigen-specific delayed-type hypersensitivity response was shown to be considerably suppressed in the tumor-bearing mice which signaled the failure of one or more components of the host-defense system. Upon the oral supplementation with RSCH, the induction of DTH response was enhanced in the 150 mg/kg/d dose group (Fig. 2) which represented an increased resistance to infection and an improved host defense capability. This demonstrates that RSCH had the potential to recruit antigen-specific T cells and modulate the T cell-mediated memory response, and thus be useful as an effective nutrient supplement with immune functions.
The blood serum thiobarbituric acid-reactive substance (TBARS) level in tumor-bearing mice was significantly higher (close to 4-fold) than in nude mice (Fig. 4). This increase in the level of TBARS indicates enhanced lipid peroxidation leading to tissue injury and failure of the antioxidative defense mechanism to prevent the formation of excess free radicals. Upon RSCH administration, the blood serum TBARS level was significantly decreased in the 100 and 150 mg/kg/d groups (Fig. 4), indicating that RSCH could effectively scavenge superoxide radicals and inhibit the generation of lipid peroxidation products in the tumor-bearing mice. Therefore, it is reasonable to hypothesize that RSCH exerted its protection against tumor formation at least partly through its antioxidative and radical scavenging properties.

In conclusion, the results of this study support the premise that RSCH could be beneficially used as a new type of dietary nutrient with beneficial functions to health. Unlike the chemicals used in chemotherapy, naturally originated food additives usually have few toxic side effects. The tumor-inhibiting effects of RSCH could be due to its antioxidative and immunological enhancement in the host as reported in this study. In medical practice, several specific nutritional supplements have already been designed that are aimed at improving the recovery of the immune function in immunocompromised patients. Based on the results of this study, RSCH could be developed as a unique food ingredient to enhance the immune functions for general or specific populations.

Acknowledgments

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References