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Identification and Characterization of Topoisomerase II Inhibitory Peptides from Soy Protein Hydrolysates

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Topoisomerases are targets of several anticancer agents because their inhibition impedes the processes of cell proliferation and differentiation in carcinogenesis. With very limited information available on the inhibitory activities of peptides derived from dietary proteins, the objectives of this study were to employ co-immunoprecipitation to identify inhibitory peptides in soy protein hydrolysates in a single step and to investigate their molecular interactions with topoisomerase II. For this, soy protein isolates were subjected to simulated gastrointestinal digestion with pepsin and pancreatin, and the human topoisomerase II inhibitory peptides were co-immunoprecipitated and identified on a CapLC- Micromass Q-TOF Ultima API system. The inhibitory activity of these peptides from soy isolates toward topoisomerase II was confirmed using three synthetic peptides, FEITPEKNPQ, IETWNPNNKP,and VFDGEL, which have IC_{50} values of 2.4, 4.0, and 7.9 mM, respectively. The molecular interactions of these peptides evaluated by molecular docking revealed interaction energies with the topoisomerase II C-terminal domain (CTD) (−186 to −398 kcal/mol) that were smaller than for the ATPase domain (−169 to −357 kcal/mol) and that correlated well with our experimental IC_{50} values ($R^2 = 0.99$). In conclusion, three peptides released from in vitro gastrointestinal enzyme digestion of soy proteins inhibited human topoisomerase II activity through binding to the active site of the CTD domain.

KEYWORDS: Topoisomerase II; soy peptides; molecular docking; dietary inhibitors

INTRODUCTION

Soybean is an important protein source and potential source of bioactive peptides. On average, 40% of the dry mass of soybeans is protein (*1*), containing a complex mixture of proteins, including seed storage proteins (*β*-conglycinin, glycinin, and albumins), enzymes (lipoxygenase, chalcone synthase, catalase, and urease), enzyme inhibitors (Bowman−Birk inhibitor and Kunitz protease inhibitors), glycosylated proteins (lectin), and peptides (lunasin). During food processing or gastrointestinal (GI) enzyme digestion, these proteins are hydrolyzed into bioactive peptides that can act as regulatory compounds with hormone-like activities (*2*). A variety of studies have shown that bioactive peptides with antihypertensive, dipeptidyl peptidase IV (DPPIV) inhibition, antithrombotic, immunostimulatory, antiamnestic, opioid, and antioxidant activities can be derived from soy proteins (*3*). Some of the targeted enzymes affected by these peptides are proteases (trypsin, papain, and chymotrypsin), peptidases [aminopeptidases and angiotensin-converting enzyme (ACE)], caspases, lipases, esterases, kinases, and topoisomerases (*4, 5*).

Among these, DNA topoisomerases are ubiquitous enzymes existing in all living organisms (*6*). The two types of eukaryotic topoisomerases, topoisomerase I and topoisomerase II, participate in cell replication and transcription by inducing transient breaks in one or both strands of the double helix to relax DNA supercoiling and, as a consequence, facilitate DNA replication. Due to the essential nature of topoisomerases in cell replication and proliferation, topoisomerase inhibitors have effectively been used to inhibit the proliferation of rapidly dividing cancerous cells. Additionally, the higher topoisomerase expression levels observed in cancer cells compared to normal cells (*7*) make topoisomerases ideal targets for a variety of anticancer agents (*8–11*). Structure-based computer screening for anticancer drugs has yielded four major topoisomerase II inhibitor chemotypes including anthracyclines, anthracenediones, anthrapyrazoles, and aureolic acids (*12*). The opening and closing of DNA molecular "gates" by topoisomerase II has been recently studied using single-molecule fluorescence microscopy experiments with these inhibitors (*13*). The results obtained suggest that the high and low fluorescence resonance energy transfer (FRET) states obtained for different topoi-

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somerase II configurations correspond to closed and open states of the DNA molecular gate.

A 43-residue glycine-rich peptide isolated from enterobacteria was identified as the first known peptide capable of inhibiting a type II DNA topoisomerase (*14, 15*). Subsequently, a cyclic depsipeptide from the culture broth of *Streptomyces* sp. A22179 was found to be a very potent topoisomerase II inhibitor with the DNA-relaxing activity of L1210 cell topoisomerase II completely inhibited at 0.08 μM (*16*). Several synthetic tetrapeptide amides have also been found to inhibit various classes of topoisomerases in vitro at subnanomolar levels (*17*). More limited information is available on the topoisomerase peptide inhibitors derived from dietary proteins that humans and other vertebrates are routinely exposed to.

One reason for this is that many problematic fractionation steps are needed to identify bioactive peptides in complex protein hydrolysates. Classical fractionation methods for these types of peptides include ion exchange, gel filtration, reverse phase HPLC, and membrane filtration with inhibitory activities being repeatedly pooled and subjected to further fractionation (*18–20*). Depending on the source of the protein isolates, combinations of different methods and multiple fractionation cycles are usually needed to generate sufficient material for bioactivity analysis. The objectives of this study were to use co-immunoprecipitation to identify inhibitory peptides in soy protein hydrolysates in a single step and to define the molecular interactions between these peptide ligands and the targeted human topoisomerase II.

MATERIALS AND METHODS

Materials. Soy protein isolate (SPI) was obtained from the Illinois Center for Soy Products, University of Illinois at Urbana-Champaign. Pepsin (EC 3.4.23.1, 662 units/mg) and pancreatin (8× USP, from porcine pancreas) were purchased from Sigma (St. Louis, MO). HPLC grade water and acetonitrile were from Fisher Chemical (Fair Lawn, NJ). Human DNA topoisomerase I and II drug screening kits and human DNA topoisomerases I and II were purchased from TopoGen (Columbus, OH). Protein A/G beads were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-human topoisomerase IIα polyclonal antibody was obtained from NeoMarkers (Fremont, CA). The identified inhibitory peptides were custom-synthesized by the American Peptide Co. (Sunnyvale, CA).

Soy Protein Hydrolysates. *Preparation of Soy Protein Hydrolysates.* In vitro digestion of SPI was performed following the procedure by Megias et al. (*21*) with several modifications. Briefly, SPI was suspended in water (1:20 w/v) and heated at 80 °C for 5 min to reduce bacterial populations and to denature endogenous lipoxygenases. Then, a sequential enzyme digestion was carried out with pepsin [EC 3.4.23.1, 662 units/mg; enzyme/flour, 1:20 (w/w); pH 2.0] and pancreatin [8× USP; enzyme/flour, 1:20 (w/w); pH 7.5] at 37 °C for 3 h each. The hydrolysis was stopped by heating at 75 °C for 20 min, and the resulting hydrolysate (GIH) was centrifuged at 27000*g* for 15 min. The supernatant was filtered through a 0.22 μm PVDF membrane and lyophilized in a FreeZone freeze-dry system (Kansas City, MO). All samples were stored at −80 °C until analysis.

Determination of Degree of Hydrolysis (DH). The DH was calculated as the ratio of the number of free amino groups and total amino groups in the sample. The number of free amino groups was determined by the trinitrobenzene sulfonic acid (TNBS) method developed by Adler-Nissen (*22*) using leucine as the standard. In the range of 0.1−0.5, the absorbance at 340 nm can be converted to leucine equivalents by the equation obtained from the standard curve: leucine equivalent (mM) $= 5.078A_{340} + 0.123$, $R^2 = 0.98$. The total number of amino groups was determined after hydrolysis of the sample with 6 N HCl at 120 °C for 24 h.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was carried out using a Pharmacia LKB-Phast System (GE Healthcare, Piscataway, NJ). Samples (3.8 μg of protein/well) were boiled for 5 min with 3 volumes of loading buffer (containing 98% of Tricine sample buffer and 2% of 2-mercaptoethanol) and loaded on a Gradient 8-25 Phast Gel (GE Healthcare). The gel was then run at 250 V, 10 mA, and 3.0 W and then fixed for 30 min in fixing solution (40% methanol, 10% acetic acid). The gel was stained with Coomassie Blue G-250 overnight and destained with 10% acetic acid. The gel picture was taken with a Kodak Image station 440 CF (Eastman Kodak Co., New Haven, CT). The molecular weight and net intensity of each band was calculated by using the Kodak ID Image analysis software, and 600 Precision Plus Protein standard was included as molecular weight marker [retention factor $= -662$ log (MW) $+ 1.585$, $R^2 = 0.99$].

Western blot analysis was carried out to determine human topoisomerase II. SDS-PAGE was run on 7.5% gel, and rabbit anti-human topoisomerase II polyclonal antibody (NeoMarkers, Fremont, CA) was used at 1:130 dilution. Horseradish peroxidase (HRP) conjugated anti-rabbit IgG (1:15000) was used as secondary antibody (Amersham Biosciences). The signal was detected using HRP substrate from an ECL Advance Western Blotting Detection Kit (Amersham Biosciences).

In Vitro Human DNA Topoisomerase I and II Catalytic Inhibitory Activity Assay. Human DNA topoisomerase I and II catalytic inhibitory activities were used as indicators to evaluate the anticancer potential of individual soy peptides. For this, lyophilized hydrolysates were suspended in water to concentrations between 0.14 and 25 mg of dry material/mL, and analysis of their inhibitory activities against topoisomerase I was conducted using a human topoisomerase I drug screening kit with modifications described previously (*23*). Reactions were assembled on ice and carried out in 20 μL volumes containing 10 mM Tris-HCl (pH 7.9), 1 mM EDTA, 150 mM NaCl, 0.1% BSA, 0.1 mM spermidine, 0.25 μg of pRYG plasmid supercoiled DNA, 2 μL (0.14−25 mg/mL) of soy isolate, soy hydrolysates or synthetic peptides, and 2 units of human topoisomerase I. Reactions were incubated at 37 °C for 45 min and terminated by adding 2 μL of 10% SDS. After that, the mixtures were incubated with 0.6 μL of proteinase K (1250 units/mL) at 50 °C for 20 min to degrade topoisomerase I, loading buffer was then added to the mixture, and DNA was extracted by adding 20 μL of chloroform/isoamyl alcohol (24:1), followed by a brief vortex and centrifugation. The extracted DNA was subjected to electrophoresis on a 1% agarose gel containing Tris/acetic acid/EDTA (TAE) buffer and run at 85 V for 240 min. Supercoiled DNA (pRYG) and relaxed DNA were included as DNA topology markers. At the completion of the electrophoresis, the gels were stained in ethidium bromide (0.5 μg/mL) for 30 min, destained for 15 min in water, and read with a Typhoon 8600 scanner (Molecular Dynamics, Sunnyvale, CA) using ImageQuant 5.1 and Fragment Analysis 1.1 image software for data analysis.

Analysis of inhibitory activity against human topoisomerase II was conducted using a topoisomerase II drug screening kit, following a similar procedure as for topoisomerase I assay. However, in this case, the reaction buffer for topoisomerase II contained 50 mM Tris-HCl (pH 8.0), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, and 30 μg/mL BSA. In addition, 4 units of topoisomerase II was used for each assay, and the reaction time was 75 min. The inhibitory activity was expressed as relative activity of topoisomerase in the presence of the tested sample in comparison to the negative control that contained no tested material. The IC_{50} was defined as the concentration needed to reduce the enzyme activity by 50% as compared to the activity in the absence of the test material, using a set of dilutions for each sample.

Purification of Human Topoisomerase II α Inhibitory Peptides by Co-immunoprecipitation. The immunoprecipitation pull-down approach developed for human topoisomerase IIα inhibitor is schematically presented in **Figure 1**. In this approach, GIH (2 mg/mL) was dissolved in reaction buffer containing 50 mM Tris-HCl (pH 8.0), 120 mM KCl, and 10 mM $MgCl_2$ and passed through a 0.2 μm PVDF membrane. Pure reaction buffer was used as a negative control. To 200 μL of each GIH sample or control sample were added 2 μL (0.4 μg/μL) of rabbit IgG and 25 μL of protein A/G beads for preclearing of nonspecific peptides possibly binding to the IgG and/or agarose beads. These samples were mixed at 4 °C for 30 min and centrifuged at 1000*g* for

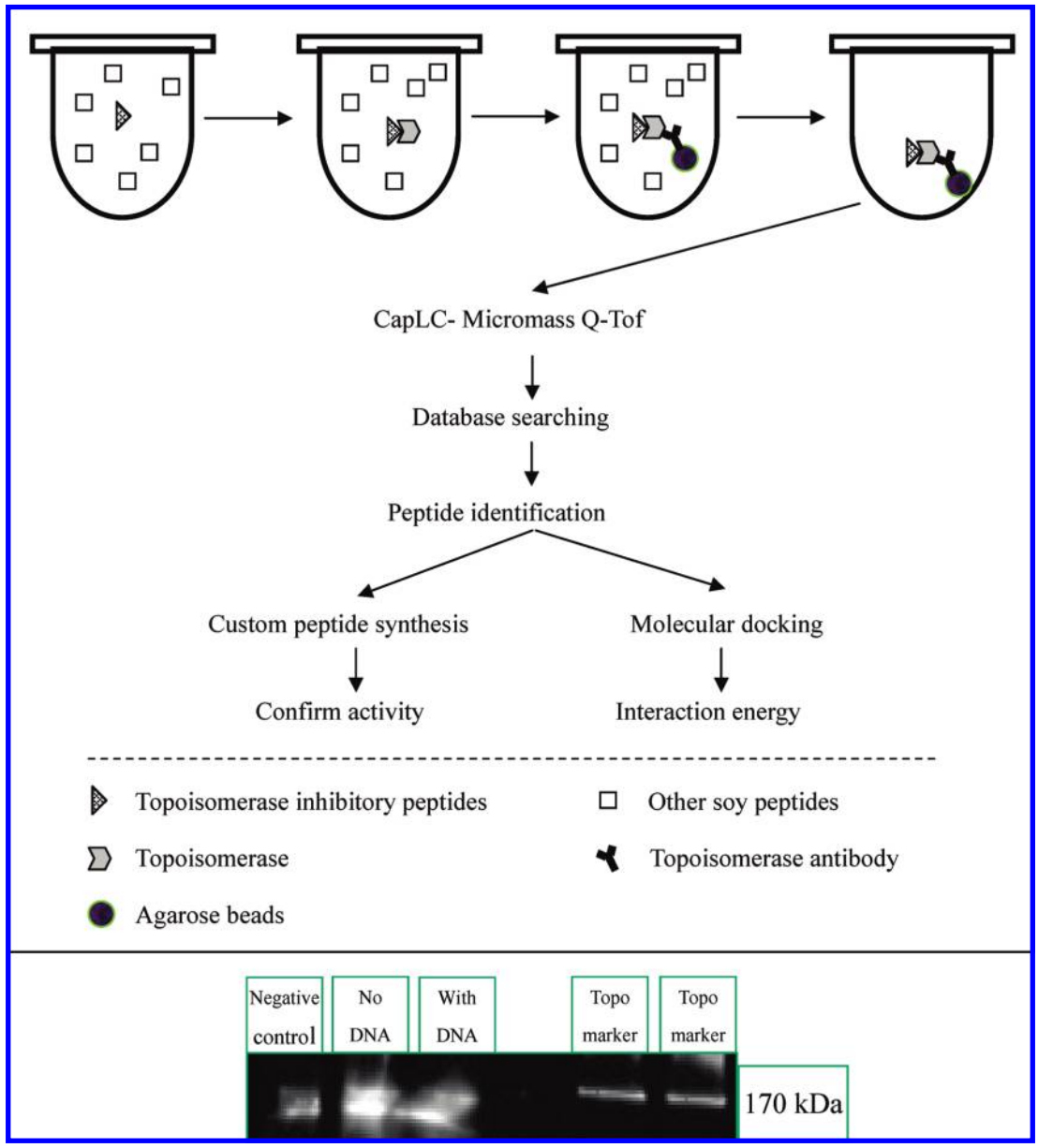

Figure 1. Identification of topoisomerase inhibitory peptides by co-immunoprecipitation approach. To identify topoisomerase inhibitory peptides from other soy peptides, topoisomerase was incubated with the soy peptide mixture and interacted with the inhibitor. Topoisomerase antibody and agarose beads were then added to pull down topoisomerase along with its inhibitors. Other soy peptides were washed away, and the topoisomerase inhibitory peptides were identified by CapLC-Micromass Q-TOF and soy protein database searching. The inhibitory activity was confirmed by topoisomerase inhibitory assay with synthetic peptides. The interaction mechanisms were explored with molecular docking with the MOE program. Western blot analysis was carried out with anti-human topoisomerase II antibody (NeoMarkers, Fremont, CA, 1:130 dilution) to confirm that topoisomerase was effectively pulled down by this procedure (shown at the bottom of this figure).

5 min at 4 °C; dithiothreitol (DTT) and ATP were added to the supernatants to final concentrations of 0.5 mM each. To evaluate the effect of DNA on the interaction of topoisomerase II and peptide inhibitors, 5 μL of pRYG DNA was added in one set of samples and labeled as hydrolysates with DNA (HD). No DNA was added to another set of hydrolysates (HN) and the negative control (NN). For all three sets (HD, HN, and NN), 10 μL of topoisomerase II (2 units/μL) was added, and samples were incubated for 30 min at 4 °C.

For immunoprecipitation, each sample was incubated with 10 μL of rabbit anti-human topoisomerase IIα polyclonal antibody (1 μg/μL) at 4 °C for 60 min and then with 30 μL of protein A/G beads for 60 min at 4 °C. The beads were centrifuged at 1000*g* for 5 min at 4 °C, and the sediment was washed twice with 50 mM Tris-HCl (pH 8.0), 120 mM KCl, and 10 mM $MgCl_2$. The sediment was resuspended in 20 μL of HPLC-grade water and boiled for 3 min to release proteins from the beads. Then, 20 μL of acetonitrile containing 0.8 μL of formic acid was added to extract the peptides and proteins, the beads were removed by centrifugation, and the final supernatant was stored at −20 °C before identification of topoisomerase inhibitory peptides. Western blot analysis was carried out with anti-human topoisomerase II antibody to confirm if topoisomerase II was effectively pulled down by this procedure.

Identification of Human Topoisomerase IIα Inhibitory Peptides. The previously co-immunoprecipitated samples were analyzed by CapLC-Micromass Q-TOF Ultima API system equipped with an Atlantis C-18 nanoAcquity 75 μm × 150 mm column (Waters, Milford, MA). A linear gradient from A (5% acetonitrile, 0.1% formic acid) to B (80% acetonitrile, 0.1% formic acid) in 60 min was used to elute peptides from the column at a flow rate of 400 nL/min. Raw mass spectra data were filtered using a Waters ProteinLynx Global Server to remove noise and to generate a peak list (PKL). To eliminate the possible peptide fractions from topoisomerase, the NN spectrum was subtracted from the HD and HN spectra. The PKL file was further processed by PEAKS 4.0 (Bioinformatics Solutions, Ontario, Canada) for de novo sequencing

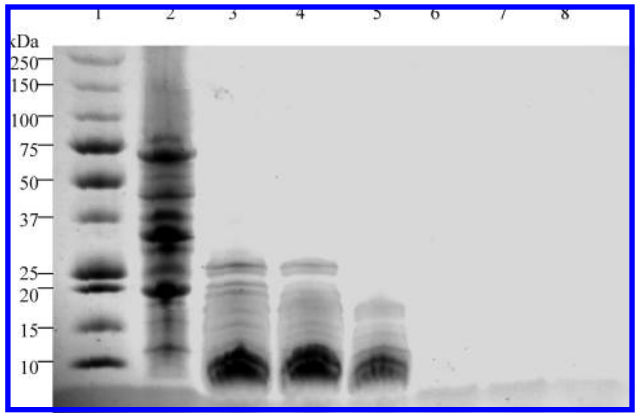

Figure 2. Time course change of protein profiles of soy protein hydrolysates. Soy protein isolate (lane 2) was hydrolyzed with pepsin for 45 min (lane 3), 90 min (lane 4), and 180 min (lane 5) and continued with pancreatin hydrolysis for 45 min (lane 6), 90 min (lane 7), and 180 min (lane 8). Samples were loaded on Gradient 8-25 Phast Gel (GE Healthcare). Precision Plus Protein standard was included as molecular mass marker (lane 1).

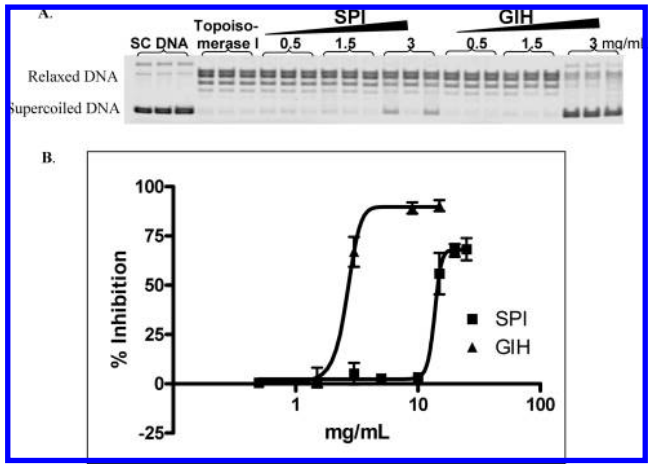

Figure 3. Comparison of topoisomerase I inhibitory activity of different concentrations of soy protein pepsin−pancreatin hydrolysate (GIH) and soy protein isolate (SPI). (**A**) Typical human topoisomerase I catalytic assay gel. Topoisomerase converts supercoiled DNA into relaxed DNA, which travels more slowly on agarose gel. With increased concentration of SPI or GIH, more supercoiled DNA was present, indicating more topoisomerase was inhibited. (**B**) After pepsin−pancreatin sequential hydrolysis, the IC_{50} decreased from 14.9 mg/mL (SPI) to 2.45 mg/mL (GIH).

and database searching. A soy (*Glycine max*) protein sequence database was compiled from entries in ExPASy databases under *Glycine max* (Swiss-Prot and TrEMBL, http://us.expasy.org/srs5/). Peptide identification was done by matching MS/MS data with the compiled soy protein sequence database, and a probability-based confidence score (range from 0 to 100%) was assigned to each peptide. To investigate the protein sources where the identified peptides were embedded, the peptide sequences were blasted against nonredundant protein sequences database under *Glycine max*, and protein sources containing 100% of each peptide sequence were recorded (*24*).

Molecular Docking of Topoisomerase Inhibitors. To investigate the interaction between human topoisomerase II and its peptide inhibitors, molecular docking was carried out using the Molecular Operating Environment (MOE) program (Chemical Computing Group, Montreal, Canada). Because an experimental structure of the C-terminal domain of human topoisomerase II was not available, we used a homology model generated from the yeast topoisomerase II structure (PDB ID: 1BGW) template that had been successfully used in structure-based virtual screening of human topoisomerase II inhibitors (*12*). This homology model was kindly provided by Serge Fermandjian (Institute Gustave Roussy, Villejuif, France) for use in this study. Yeast topoisomerase II ATPase structure was downloaded from the Protein Data Bank (PDB ID:1QZR) (*25*). Prior to molecular docking, hydrogen was added to each structure, and then the topoisomerase II structure was energy-minimized using the CHARMm22 force field (*26*) after fixing of the heavy atoms. The structures of the FEITPEKNPQ, IETWNPNNKP, and VFDGEL peptides were built using the PROTEIN BUILDER function in MOE and energy-minimized with CHARMm22 force field. The peptides were docked against the CTD and ATPase domains of topoisomerase II using the DOCK function in MOE that uses a simulated annealing search protocol to find favorable binding conformations between a small, flexible ligand and a rigid target protein. Docking parameters were set to the MOE default values of 25 simulated annealing runs and 6 Monte-Carlo cycles per run. The interaction energies between the ligand conformation and the target proteins were calculated using a built-in potential function in the DOCK function, and the lowest energy conformation after each run was saved. The docking results were later sorted on the basis of total energy and the best pose that had lowest total energy and made biological sense, and the ligand−protein complex was energy-minimized using the CHARMm22 force field in MOE. Interaction energies between the minimized protein and the ligand were calculated as the difference between the total potential energy of the minimized complex and the sum of the individual protein and ligand components of the complex (*27, 28*). The potential energy function contains the sum of the ligand/protein internal energy, van der Waals, and electrostatic energy terms.

Statistical Analysis. The sigmoidal dose−response analysis of the concentration needed to inhibit 50% (IC_{50}) of topoisomerase was determined by nonlinear regression (curve fit) using the GraphPad Prism software. The difference between IC_{50} of each sample was evaluated by one-way ANOVA with Tukey's multiple-comparison test, and $p < 0.05$ was considered to be significant. Data were analyzed by one-way ANOVA. All experiments were repeated at least three times, and the means and standard deviations were calculated.

RESULTS AND DISCUSSION

Pepsin−Pancreatin Hydrolysis Increased Human Topoisomerase I and Topoisomerase II Catalytic Inhibitory Activities. Figure 2 presents the soy protein profile change during the in vitro enzyme digestion. The molecular masses of proteins in the nonhydrolyzed soy protein isolate (lane 2) ranged from 10 to 75 kDa with the α′ (69 kDa), α (62 kDa), and β (45 kDa) subunits of β-conglycinin and acidic (33 kDa) and basic (20 kDa) chains of glycinin clearly seen in the picture. After 45 min of pepsin digestion (lane 3), all proteins larger than 30 kDa were digested into smaller proteins and peptides at the molecular masses of 8−12 kDa. Increases in the hydrolysis time from 45 to 90 min (lane 4) did not show any significant change in the protein profile. Another 90 min of digestion with pancreatin (lane 7) hydrolyzed all proteins larger than 8−12 kDa to even smaller peptides, which are believed to have diffused out of the gel. Further pancreatin digestion rapidly cut proteins and peptides into fragments of <3 kDa, as determined by gel filtration chromatography (data not shown) and agreeing with previous findings (*29*). Only very faint bands of these smaller peptides can be seen on this gel.

To evaluate the inhibitory effect of enzymatic digestion and peptide formation on topoisomerase I activity, soy protein samples before (SPI) and after (GIH) sequential digestion were incubated with various concentrations of topoisomerase I. **Figure 3A** shows a typical topoisomerase I result in which topoisomerase activity converts supercoiled DNA into relaxed DNA that travels more slowly on agarose gels. With increased concentrations of SPI or GIH, more supercoiled DNA is present, indicating that topoisomerase activities are inhibited. The dose−response inhibitions of SPI and GIH shown in **Figure**

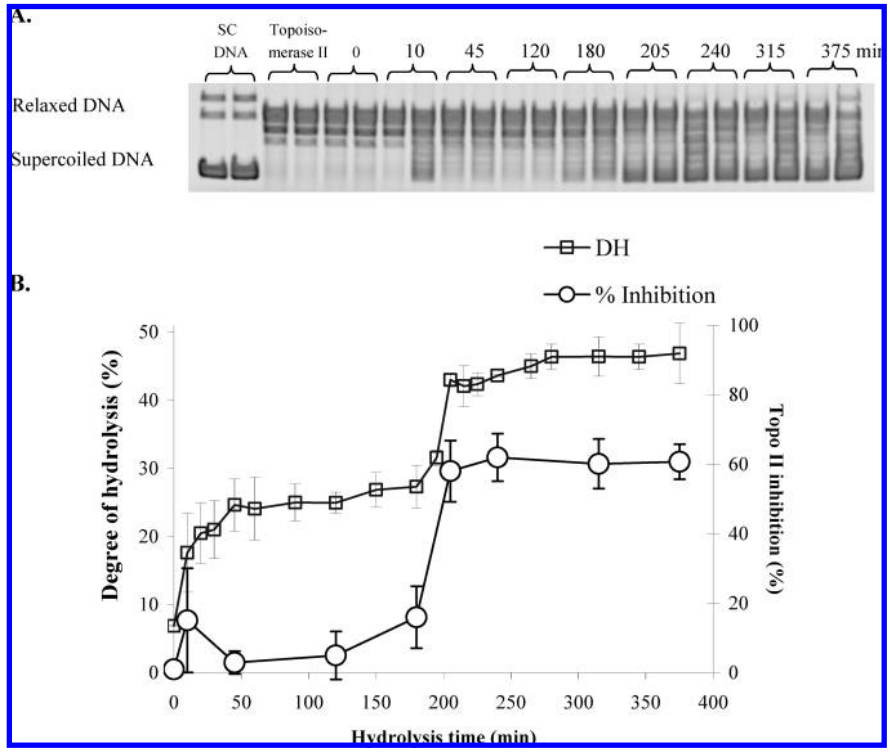

Figure 4. Time course changes of topoisomerase II inhibitory activities during sequential pepsin−pancreatin hydrolysis. (**A**) Typical human topoisomerase II catalytic assay gel. Soy proteins were hydrolyzed with pepsin from 0 to 180 min at 37 °C and pH 2.0, followed by pancreatin hydrolysis from 195 to 375 min at pH 7.5. Topoisomerase converts supercoiled DNA into relaxed DNA, which travels more slowly on agarose gel. With increased hydrolysis time, more supercoiled DNA was present, indicating more topoisomerase was inhibited. (**B**) Time course changes of topoisomerase II inhibitory activities at 0.5 mg/mL and degree of hydrolysis (DH) of soy proteins during sequential pepsin−pancreatin hydrolysis. The DH was monitored by TNBS assay at different time points of the hydrolysis.

Table 1. Human DNA Topoisomerase II Catalytic Inhibitory Peptides Identified from Soy Protein Hydrolysates by an Immunoprecipitation Approach[a]

sample	peptide	mass (exptl)	M_r (calcd)	confidence score (%)	protein source
HD/HN	FEITPEKNPQ	1201.66	1201.60	98.3	β-conglycinin α, α', β subunit
HD/HN	IETWNPNNKP	1211.66	1211.59	95.9	glycinin G1, G2, G3 subunit
HD/HN	VFDGEL	678.36	678.32	99.0	glycinin G1, G3, G4 subunit
HD	EPQQPGEKEEDEDEQPRPI	2249.10	2249.01	99.0	β-conglycinin α subunit
HN	IETWNPNNKPF	1358.72	1358.66	88.3	glycinin G1, G2, G3 subunits
HN	LAGNPDIEHPET	1291.65	1291.60	99.0	glycinin G5 subunit

[a] Peptides purified in the presence of DNA (HD) or without DNA (HN) by co-immunoprecipitation were loaded on a CapLC-Micromass Q-TOF Ultima API system equipped with an Atlantis C-18 nanoAcquity 75 μm × 150 mm column. Peptide identification was done by matching MS/MS data with the compiled soy protein sequence database, and a probability-based confidence score (range from 0 to 100%) was assigned to each peptide by PEAKS software. To investigate the protein sources where the identified peptides were embedded, the peptide sequences were blasted against nonredundant protein sequences database under *Glycine max* and protein sources containing 100% of each peptide sequence were recorded.

3B clearly indicate that the IC_{50} value for topoisomerase I inhibitory activities were decreased from 14.9 mg/mL (before hydrolysis, SPI) to 2.45 mg/mL (after hydrolysis, GIH) by enzyme digestion. This indicated that the small peptides produced by simulated gastrointestinal digestion contributed to the inhibitory activity on topoisomerase I.

The change of inhibitory activity of soy proteins on topoisomerase II during the course of pepsin−pancreatin hydrolysis is shown in **Figure 4**. It shows that after the addition of pepsin at 0 min, the degree of hydrolysis (DH) increased rapidly and reached a plateau at 24.6% DH at 45 min. In the following 135 min, the degree of pepsin hydrolysis increased only slightly (3%). Addition of pancreatin, at 180 min, increased hydrolysis to 43.0% after 20 min. This correlates with **Figure 2**, which shows a change in protein profiles 45 min after the addition of pancreatin. At the end of the sequential enzyme digestion procedure, the average DH was 46.9%. In this time period, the inhibitory activity on topoisomerase II of the SPI (0.5 mg/mL) changed with the degree of pepsin hydrolysis from no topoisomerase II inhibition to 5% at 120 min and 16% at 180 min. Together with the sharp increase in DH after the addition of pancreatin, a dramatic increase in inhibitory activity on topoisomerase II was observed (58% at 25 min after the addition of pancreatin to finally 61% at 360 min at the end of the sequential digestion). The topoisomerase II inhibitory activity followed a pattern similar to that of DH, indicating that the formation of small peptides is important to the inhibitory activity on topoisomerase II. Furthermore, the relatively low activity of pepsin hydrolysates indicated that peptides larger than 10 kDa may be less active than the smaller peptides obtained in these digestions (**Figure 2**).

To the best of our knowledge, this is the first time that inhibition of topoisomerase activities by food protein hydrolysates has been reported. Previously, studies on ACE inhibitory activities of sunflower and soy proteins during the time course of pepsin−pancreatin hydrolysis have shown increased enzyme

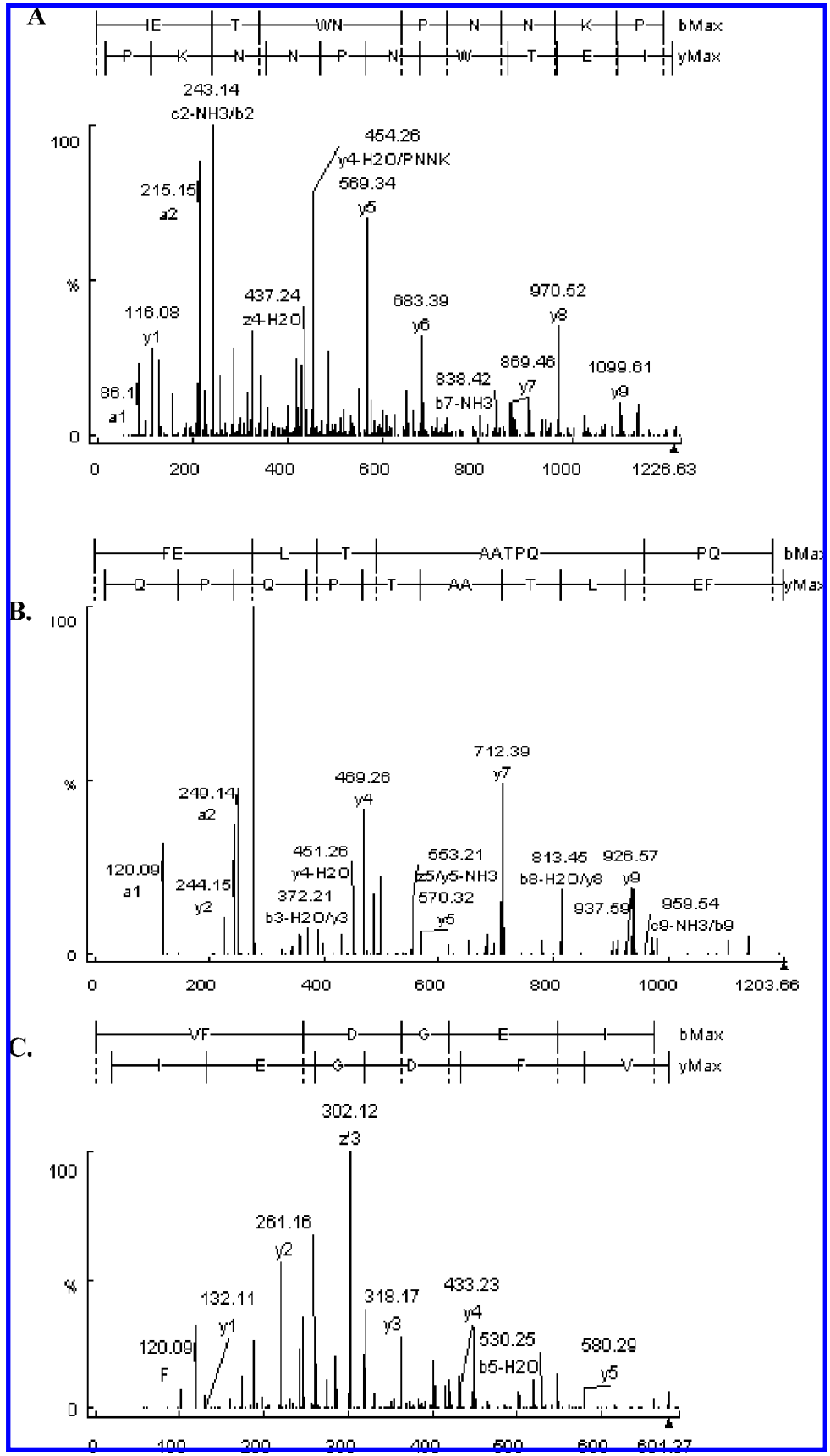

Figure 5. MS/MS profiles of peptide IETWNPNNKP (**A**), FEITPEKNPQ (**B**), and VFDGEL (**C**). Peptides purified by co-immunoprecipitation method were loaded on a CapLC-Micromass Q-TOF Ultima API system equipped with an Atlantis C-18 nanoAcquity 75 μm $\times$ 150 mm column. The peptide sequences were determined by matching MS/MS profile with *Glycine max* protein database with PEAKS software.

inhibitory activities (*21, 29*). However, in these studies, the ACE inhibitory activity reached a maximum at the end of pepsin digestion or at the early stage of pancreatin digestion and decreased with further pancreatin digestion (*21, 29*). These

Table 2. Human DNA Topoisomerase II Inhibitory Activities and Interaction Energy of Molecular Docking of Three Peptides against Human Topoisomerase II CTD Domain and ATPase Domain[a]

topoisomerase II inhibitor	interaction energy (kcal/mol) CTD domain	interaction energy (kcal/mol) ATPase domain	IC_{50} (mM) of synthetic peptides
FEITPEKNPQ	−398.1	−254.9	2.4
IETWNPNNKP	−355.0	−356.8	4.0
VFDGEL	−186.0	−168.7	7.9

[a] The interaction energy was calculated using the potential energy function in MOE. The lower the energy, the stronger the binding is. The IC_{50} values of peptides were determined by cytotoxicity assay with custom synthetic peptides.

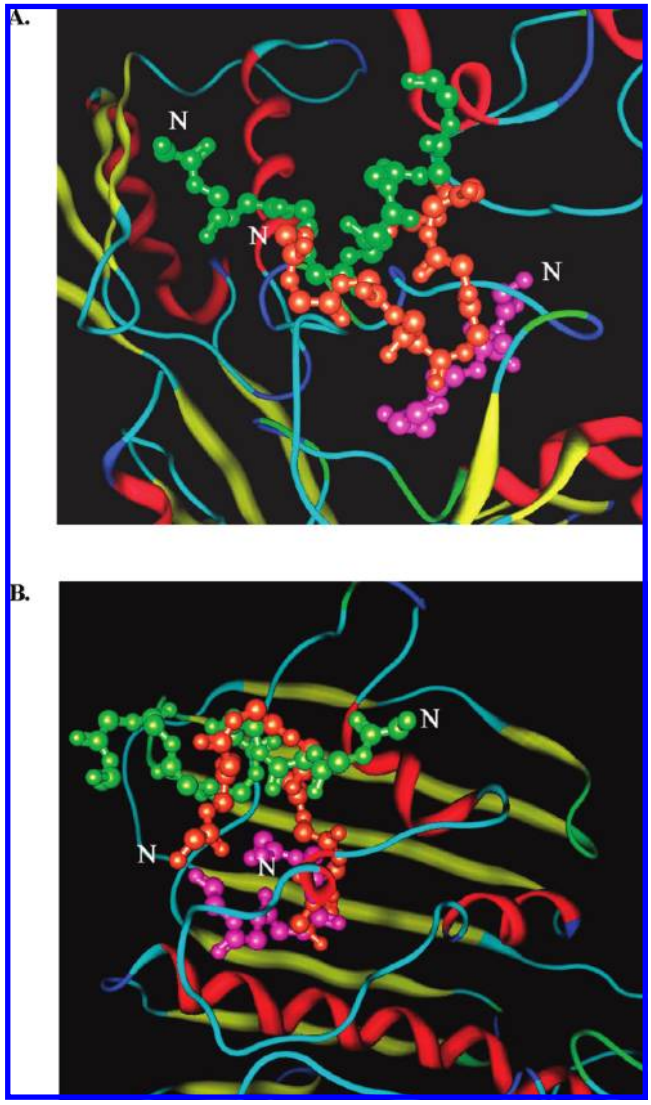

Figure 6. Binding orientation of soy peptides with human topoisomerase CTD (**A**) and ATPase domains (**B**). The N terminus of each peptide is labeled with an N, and peptides are designated IETWNPNNKP (orange), FEITPEKNPQ (green), and VFDGEL (purple).

differences in the behavior of topoisomerase and ACE inhibitory activities may be explained by variations in the overall structures of these proteins, the conformations of their active sites, and/or their affinities for different terminal amino acids.

GIH inhibited 50% of topoisomerase I activity at 2.45 mg/mL and 61% of topoisomerase II activity at 0.5 mg/mL, indicating that GIH is more potent as a human topoisomerase II inhibitor than a topoisomerase I inhibitor (**Figures 3** and **4**). This topoisomerase specific inhibition, which is not uncommon,

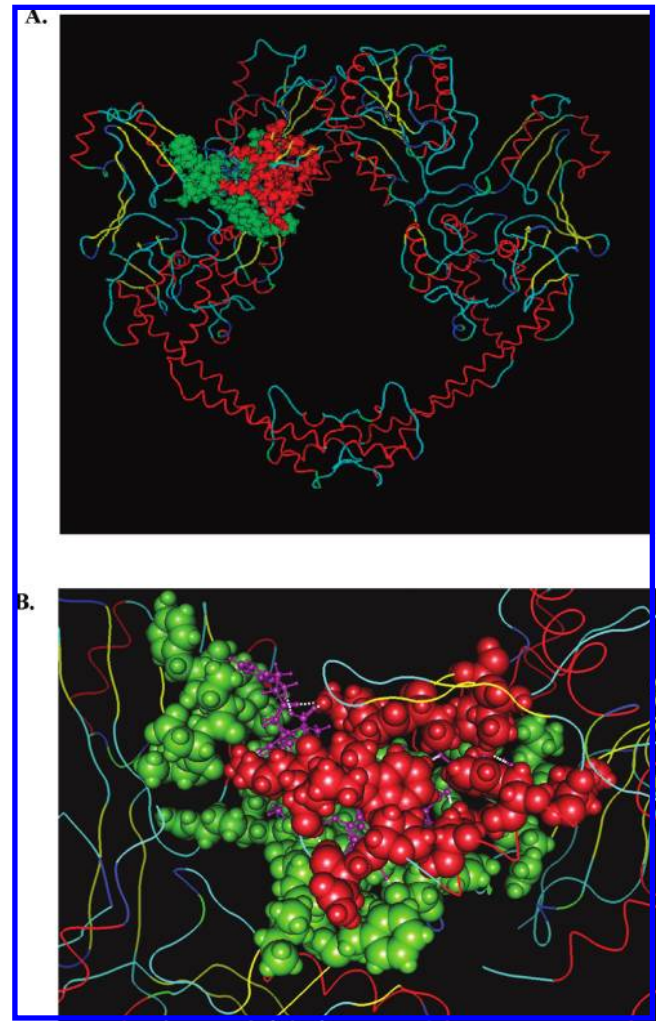

Figure 7. Docking of the FEITPEKNPQ peptide in the active site of the CTD domain of topoisomerase II. (**A**) The full view of the FEITPEKNPQ structure is rendered in purple ball-and-stick format with its adjacent CTD side chains shown in red and green space-filling format. (**B**) The magnified view of the peptide ligand is shown in ball-and-stick format with side chains in topoisomerase II within a radius of 10 Å of the ligand rendered in space-filling format. The topoisomerase II backbone is rendered in line form, with hydrogen bonds shown with dotted lines.

has previously been observed for a DNA gyrase inhibitor obtained by optimization of a pyrazole derivative; this compound inhibits human topoisomerase II but not topoisomerase I (*30*). These results prompted us to focus further analysis on topoisomerase II inhibitory peptides.

Identification of Topoisomerase II Inhibitory Peptides by Co-immunoprecipitation (CIP). Because there are thousands of peptides in protein hydrolysates and fractionation steps are generally required for the identification of bioactive peptides, previous studies have used anion exchange columns, membrane fractionations, RP-HPLC, and gel filtration to isolate ACE inhibitory peptides (*29*). Despite sequential gel filtration or membrane separation, preparative RP-HPLC, and analytical RP-HPLC, the fractions of interest in these and other studies still contained up to 15 peptides (*31–33*). Newer approaches for these purifications have employed ACE-immobilized affinity chromatography to purify ACE inhibitory peptides from sunflower and soy glycinin hydrolysates (*34, 35*), obviously shortening the purification steps and more selectively isolating the relevant peptide fragments. In the current study, we also took advantage of the specific affinity between an enzyme and its inhibitory

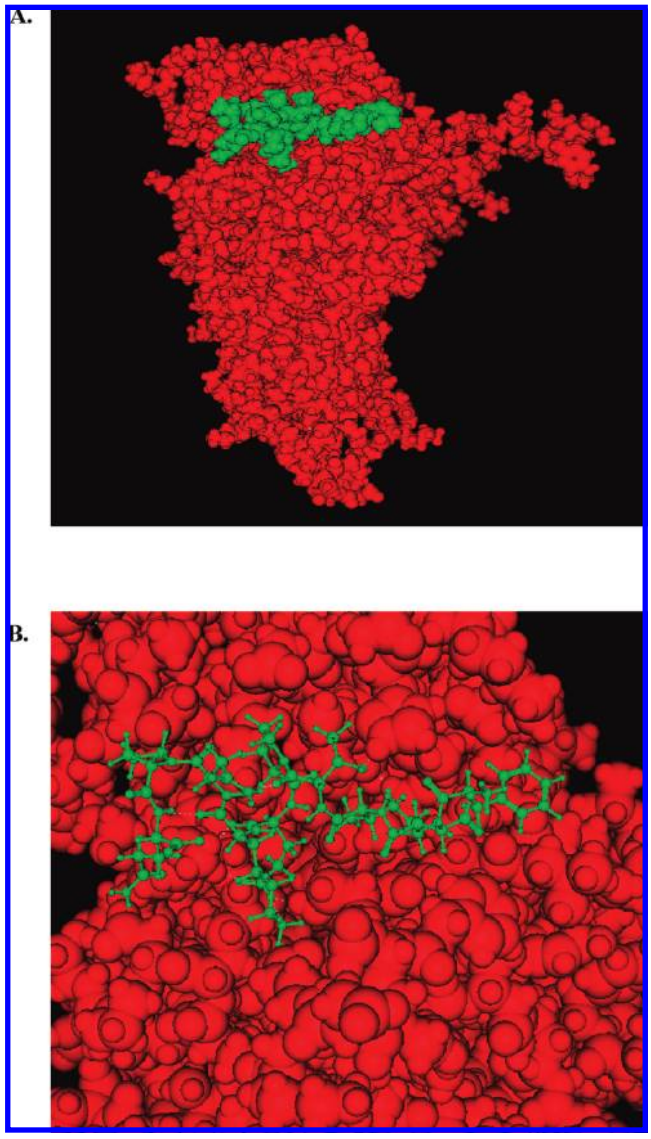

Figure 8. Docking of the FEITPEKNPQ peptide in the active site of the ATPase domain of topoisomerase II. As in **Figure 6**, (**A**) shows the full view and (**B**) shows the magnified view; the ATPase domain is shown in red and the FEITPEKNPQ peptide in green.

peptides but, instead of immobilizing enzyme on activated agarose gels, we attached the topoisomerase II antibody to agarose beads and isolated topoisomerase II and its interacting peptides using simple centrifugation procedures. Under these conditions, only 50−500 ng of topoisomerase II are needed, making it an appealing method for expensive and difficult to isolate enzymes such as human topoisomerase II.

Western blot analysis, shown at the bottom of **Figure 1**, indicated that topoisomerase II was successfully immunoprecipitated with the topoisomerase II antibody attached to agarose beads. Subsequent analysis of the low-abundance topoisomerase II inhibitory peptides pulled down by CIP indicated that Nano-LC-Q-TOF analysis was more sensitive than LCQ analysis and allowed peptide sequencing in the low-femtomole and attomole ranges (*36, 37*).

To obtain reliable peptide sequencing results, enzyme specificity and source protein database were chosen in the PEAKS software. However, the enzymes used in this study, pepsin and pancreatin, were not very specific, and also the soy protein database was not in the software. Pepsin hydrolyzes peptide bonds with Trp, Tyr, Phe, Leu, Ile, or Val (*38*). Pancreatin is a

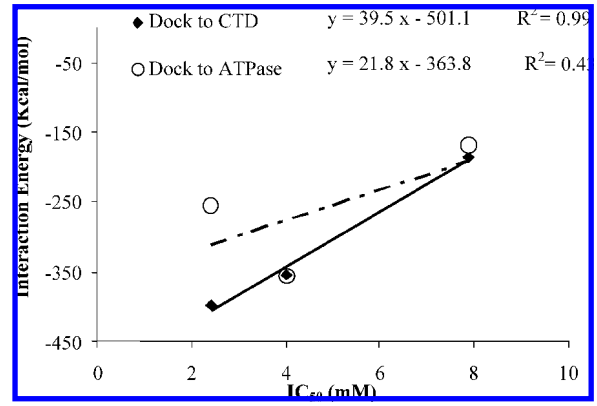

Figure 9. Correlation between topoisomerase II inhibitory activities and interaction energies predicted from molecular docking of the three peptides with the CTD and ATPase domains of topoisomerase II. The interaction energies are as calculated using the MOE program. The IC_{50} values of peptides were determined by cytotoxicity assay with custom-synthesized peptides.

mixture of pancreatic enzymes, including trypsin, chymotrypsin, elastase, and carboxypeptidase, as well as amylase and lipase (*39*). On the basis of this information, pepsin−pancreatin digestion profiles were added to the PEAKS software.

The soy protein sequence database was compiled using the ExPASy databases including the amino acid sequences of all major soy proteins (*40*). **Table 1** presents the peptide sequences identified from the CIP approach with >80% confidence scores and their protein sources. The negative control (NN), as expected, did not give any peptide sequence with a confidence score of >60%. Three peptides, FEITPEKNPQ, IETWNP-NNKP, and VFDGEL, were identified in both HD and HN samples with confidence scores of >95%. Blast results indicated that FEITPEKNPQ can be found in the α, α′, and β subunits of β-conglycinin, whereas IETWNPNNKP and VFDGEL can be found in the G1, G2, G3 and G1, G3, G4 subunits of glycinin, respectively. These three peptides were identified in samples with and without DNA, indicating that they are involved in a DNA-independent direct interaction with topoisomerase II.

One peptide from the α subunit of β-conglycinin, EPQQPGE-KEEDEDEQPRPI, was identified only in HD, and two glycinin-embedded peptides, IETWNPNNKPF and LAGNPDIEHPET, were found only in HN. The IETWNPNNKPF contains the entire sequence of IETWNPNNKP that was identified in both HD and HN as mentioned above. The MS/MS profiles of the three peptides identified in both with and without DNA samples are presented in **Figure 5**. The clear fragmentation of parent ions together with database searching allowed unambiguous identification of target peptides.

Soy protein is a complex mixture containing many seed storage proteins, enzymes, enzyme inhibitors, and other proteins (*41*). The fact that all of the peptides identified in this study were from soy β-conglycinin and glycinin (acid and basic) and often represented in multiple subunits explains the high abundance of these topoisomerase inhibitory peptides because β-conglycinin and glycinin constitute 65−80% of the total soy seed protein pool (*41*).

Molecular Interaction of Human Topoisomerase II and CIP Identified Peptides. Two approaches were taken to understand the interaction of human topoisomerase II with the CIP identified peptides. In the first, molecular modeling and docking were used to provide information on potential interactions between topoisomerase II and its ligands. Although the complete structure of human topoisomerase II has not been

defined from a crystal structure, it is clear that human topoisomerase II contains CTD and ATPase domains and presents a high sequence similarity to the sequences of *Saccharomyces cerevisiae* topoisomerase II. Human and *S. cerevisiae* DNA topoisomerase II sequences exhibited an identity of 47.3% and a similarity of 68.2% (*12*). The availability of structures for both *Escherichia coli* DNA gyrase and *S. cerevisiae* topoisomerase II provided a nearly complete view of the structure of topoisomerase IIα (*42–46*). The ATPase domain of human topoisomerase IIα (PDB ID:1ZXM) (*44*), aligned very well with that from *S. cerevisiae* (PDB ID:1PVG). From the details provided in Wei et al. (*47*), Schoeffler et al. (*48*), and Dal Ben (*49*), topoisomerase II can cut double strands of G-segment (gate segment) DNA in the presence of ATP and allow the T-segment (transfer segment) DNA to pass through it. The G-segment is subsequently rejoined and, in this process, supercoiling in the DNA is relaxed. During the reaction, the G-segment DNA initially binds to the CTD domain of topoisomerase II and the ATPase domain opens. Then a T-segment is captured by the ATPase domain upon ATP binding and dimerization of ATPase domain. Pi will be released from ATP hydrolysis and triggers the G-segment cleavage at both strands. This allows the T-segment passage through the G-segment. Then the C-gate will open to release the T-segment out of the CTD domain, whereas the G-segment will ligate back.

It is clear that both the ATPase and CTD domains are important for enzyme function. In this study, the three peptides identified in HN and HD, FEITPEKNPQ, IETWNPNNKP, and VFDGEL, were docked individually with each of these domains. Because the crystal structure of the CTD domain of human topoisomerase II is not yet available, docking simulations were carried out with a homology model of the CTD domain (*12*) and the crystal structure of the ATPase domain (*48*). This process indicated the three peptides can be docked in the active sites of both domains of topoisomerase II with the very low interaction energies shown in **Table 2**. The longer peptides (FEITPEKNPQ and EITPEKNPQ) were predicted to occupy the same location in the CTD domain. The shorter peptide (VFDGEL) was predicted to bind slightly toward the C terminus of the active site. The longer peptides were predicted to bind in the same orientation, whereas the VFDGEL peptide bound in the opposite orientation (**Figure 6A**). In the ATP subunit, all three peptides occupied the same cavity but were predicted to bind in different orientations (**Figure 6B**).

As an example, the docking results of the FEITPEKNPQ peptide to the CTD and ATPase domains of topoisomerase II are presented in **Figures 7** and **8**, respectively. **Figure 7A** presents the full view of the predicted binding conformation of FEITPEKNPQ in one of the two identical and symmetrical active sites in the CTD domain of topoisomerase II. **Figure 7B** presents a magnified view of the predicted EITPEKNPQ binding conformation fitting into the active cavity of the CTD domain through hydrogen binding to the active loop of the helix−turn−helix motif (HTH) domain. In the topoisomerase reaction, double-stranded G-segment DNA binds in the active site of CTD domain in such a way that the hydroxyl group of the active site Tyr805 will interact with DNA and form an ester bond with 5′-phosphates of the DNA (*50*). Peptides binding in the active site and interfering with these interactions obviously would alter the topoisomerase II active site structure and, therefore, affect enzyme activity.

As outlined in Wei et al. (*47*), G-segment DNA binds to the topoisomerase II dimer at the second gate of the cleavage−reunion core. An incoming T-segment of DNA is captured in the upper cavity between the upper and middle gates by ATP binding-promoted dimerization. Hydrolysis and release of one of the two bound ATP molecules to ADP and Pi lead to an asymmetric retraction of the catalytic Lys378, opening of the enzyme-bridged middle gate of the protein DNA complex, and movement of the T-segment from the upper cavity formed in the ATPase domain into the lower cavity formed by the cleavage−reunion core. Hydrolysis of the remaining ATP and opening of the bottom gate allows the T-segment to egress from the complex and resets the system.

When ATP binds to the ATPase domain and T-segment DNA is trapped between the ATPase subunits, the two subunits dimerize by interlocking the tail protein chain of one subunit with the dimerization binding cavity of the other subunit (*48*). As shown in **Figure 8A**, which presents the full view of the predicted FEITPEKNPQ binding mode in one of the two identical ATPase subunits, and in **Figure 8B**, which shows FEITPEKNPQ docking in the ATP binding site and dimerization cavity, it is likely that this peptide blocks ATP binding and ATPase dimerization.

Calculations of the interaction energies between each of these peptides and topoisomerase II summarized in **Table 2** predict that FEITPEKNPQ has the strongest binding to CTD domain (−398.1 kcal/mol), followed by IETWNPNNKP (−355.0 kcal/mol) and VFDGEL (−186.0 kcal/mol). The peptides are generally predicted to have weaker interactions with the ATPase domain than with the CTD domain, as reflected by the higher interaction energies for IETWNPNNKP (−356.8 kcal/mol), FEITPEKNPQ (−254.9 kcal/mol), and VFDGEL (−168.7 kcal/mol). To understand the correlation between their docking scores and topoisomerase inhibitory activities, these three peptides were custom-synthesized and their topoisomerase II inhibitory activities were tested. As shown in **Table 2**, the IC_{50} values of FEITPEKNPQ, IETWNPNNKP, and VFDGEL against topoisomerase II were 2.4, 4.0. and 7.9 mM, respectively. The correlation between topoisomerase II inhibitory activities and interaction energy of molecular docking of the three peptides against topoisomerase II CTD domain and ATPase domain are presented in **Figure 9**. The topoisomerase II inhibitory activity correlates very well with the docking results with CTD domain ($R^2 = 0.99$). The correlation between topoisomerase II inhibitory activity and docking to ATPase domain was not significant ($R^2 = 0.43$).

It can be concluded that the three peptides released from in vitro gastrointestinal enzyme digestion of soybean proteins inhibit topoisomerase II activity through binding to the active site of the CTD domain. The implications of this research are to improve the understanding of the mechanism of action of soy bioactive components, particularly soy peptides. Even though in vitro assays may not have translational physiological implications, these findings provide basic information for the understanding of active peptide mixtures. Further in vivo studies may lead to the elucidation of the role of DNA topoisomerase II inhibitors in carcinogenesis. More data on peptide bioavailability are also needed to conclusively determine their influence on human health.

ABBREVIATIONS USED

CIP, co-immunoprecipitation; CTD, C-terminal domain; G-segment, gate segment; IC_{50}, concentration needed to inhibit 50% of topoisomerase activity; PDB, Protein Data Bank; Q-TOF, quadrupole-time-of-flight; sc-DNA, supercoiled DNA; T-segment, transfer segment.

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