Published in final edited form as:

Mol Nutr Food Res. 2010 August ; 54(8): 1202–1209. doi:10.1002/mnfr.200900106.

Heat-solubilized curry spice curcumin inhibits antibody-antigen interaction in *in vitro* studies: A possible therapy to alleviate autoimmune disorders

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Abstract

Chronic and complex autoimmune diseases, currently treated palliatively with immunosuppressives, require multi-targeted therapy for greater effectiveness. The naturally occurring polyphenol curcumin has emerged as a powerful “nutraceutical” that interacts with multiple targets to regress diseases safely and inexpensively. Upto 8 g/day of curcumin for 18 months was non-toxic to humans. However, curcumin's utility is limited by its aqueous insolubility. We have demonstrated a heat-mediated 12-fold increase in curcumin's aqueous solubility. Here we show by, SDS-PAGE and SPR, that heat-solubilized curcumin binds to proteins. Based on this binding we hypothesized that heat-solubilized curcumin or turmeric would prevent autoantibody targeting of cognate autoantigens. Heat-solubilized curcumin/turmeric significantly decreased binding of autoantibodies from Sjögren's syndrome (SS) (up to 43/70 % respectively) and SLE (up to 52/70 % respectively) patients as well as an animal model of SS (up to 50/60 % respectively) to their cognate antigens. However, inhibition was not specific to autoimmunity. Heat-solubilized curcumin/turmeric also inhibited binding of polyclonal anti-spectrin to spectrin (50/56 % respectively). Thus, we suggest that the multifaceted heat-solubilized curcumin can ameliorate autoimmune disorders. In addition, the non-toxic curcumin could serve as a new protein stain in SDS-PAGE even though it is less sensitive than the Coomassie system which involves toxic chemicals.

Keywords

Curry spice; curcumin; turmeric; *Curcuma longa*; nutraceutical; autoimmunity; antioxidant; solubility; SDS-PAGE; protein staining

1. Introduction

Treatments for chronic diseases need multi-targeted therapy. Immunosuppressives have been the main therapeutic choice for chronic and complex diseases like Sjögren's syndrome (SS) and systemic lupus erythematosus (SLE) and treatment is largely palliative [1,2]. SS is characterized by dry eyes and dry mouth, presence of anti-Ro 60/anti-La autoantibodies in up to 90% of patients and a highly significant over representation of lymphoma compared to

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Conflict of interest statement: There is no conflict of interest for all authors.

the normal population [3]. SLE is also a multisystem disorder distinguished by antibodies to a variety of self-proteins, often affecting kidney function and involving premature atherosclerosis. Anti-Ro autoantibodies are present in up to 50% of SLE patients [4].

The naturally occurring phytochemical curcumin (CU), the most active component in the Indian curry spice turmeric (TU) (*Curcuma longa*), has emerged as a “nutraceutical” that can interact with multiple targets to regress diseases safely and inexpensively. In addition to inhibiting tumorigenesis, metastasis, platelet aggregation, inflammatory cytokine production, cataract formation, inflammatory bowel disease and myocardial infarction, CU has been shown to lower cholesterol, suppress diabetes, enhance wound healing, modulate multiple sclerosis and Alzheimer's disease and block HIV replication [5-9]. Even though 12 g/day was found to be non-toxic to humans its insolubility limits its biological utility. Our laboratory has shown that heat treatment increased CU's solubility 12-fold in water without affecting its integrity. Moreover, heat-solubilized CU inhibited 4-hydroxy-2-nonenal mediated oxidative modification of a protein substrate by 80%.

Since we were able to heat-solubilize curcumin in water, we were able to find out that aqueous curcumin stained proteins on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). Based on this observation, that heat-solubilized curcumin could bind to proteins on SDS PAGE, we hypothesized that it would bind to antigen and/or antibody and thus inhibit autoantibody-antigen interaction. Such an intervention would be of therapeutic potential to prevent autoantibody targeting of cognate antigens and thus ameliorate autoimmune disorders such as SS and SLE.

2. Materials and Methods

2.1. Instrumentation and Materials

The BIAcore instrument, part of Oklahoma Medical Research Foundation (OMRF) Core Facility, was from GE Healthcare, Piscataway, NJ, USA. Carboxy methylated dextran-5 sensor chips (research grade), P20 surfactant and amine coupling kit were also from GE Healthcare. Serocluster “U” vinyl ELISA plates were from Costar, Cambridge, MA, USA.

Purified Ro 60 (SS-A) was purchased from Immunovision, Springdale, AK, USA. Recombinant human La (SS-B) was a gift from Dr. Michael Bachmann, Dresden, Germany. A peptide spanning the Ro 60 [10,11] sequence 273-289 (LQEMPLTALLRNLGKMT) tagged with a terminal cysteine was synthesized as a multiple antigenic peptide (MAP) [12] and a linear peptide at the Molecular Biology Resource Facility, University of Oklahoma Health Sciences Center by a manual stepwise solid phase procedure. Curcumin and phosphate buffered saline tablets were from Sigma Chemical Company, St. Louis, MO, USA. Turmeric was purchased from a local grocery. Alkaline phosphatase conjugates were from Jackson Laboratories, Bar Harbor, ME, USA. All other chemicals were reagent grade.

2.2. Patients

Primary SS and SLE sera were kindly provided by Dr. Tom Gordon, Flinders Medical Institute, Adelaide, Australia and the Lupus Family Registry and Repository, OMRF, Oklahoma City, Oklahoma, USA respectively.

2.3. Heat-solubilization of curcumin and turmeric

Curcumin or turmeric (5 mg/ml) was solubilized as described [13]. Essentially hot distilled water was added to either curcumin or turmeric at 5 mg/ml (1 ml hot distilled water/ mg curcumin or turmeric) and further boiled for 10 minutes and centrifuged twice. The

supernatant was used to test inhibition of binding of anti-Ro 60 or anti-Ro 273 peptide antibodies binding to their cognate antigen by ELISA.

2.4. Mice immunization

Ten BALB/c mice were used in this experiment. Five mice were immunized (day one) with 50 µg of a linear peptide (273-289 amino acid sequence from the Ro 60 autoantigen) emulsified in Freund's Complete Adjuvant (FCA). Five mice were administered saline in FCA. Subsequent boosts were in Freund's Incomplete Adjuvant. The first, second and third boosts were given on day 7, 27 and 60 respectively [14]. The research was approved by the Institutional Review Board and was conducted in accordance with the internationally accepted principles for laboratory animal use.

2.5. Ro 60 or Ro 273 multiple antigenic peptide ELISA

Enzyme linked immunosorbant assay (ELISA) was carried out as mentioned earlier with modifications. Purified Ro 60 or Ro 60 multiple antigenic peptide (MAP) was coated at 5 µg/well at room temperature for two hours. The plate was washed twice with phosphate buffered saline and tapped dry. Heat-solubilized curcumin (7.4 µg/ml) or turmeric (15.5 µg/ml) was added to each well (100 µl) except those wells receiving uninhibited sera samples. Purified water (100 µl) was added to each of the wells to which only sera was to be added (uninhibited samples). In individual microcentrifuge tubes, 2.4 µl of each serum sample were added to 40 µl of heat solubilized curcumin (7.4 µg/ml) or turmeric (15.5 µg/ml). The plate and the sera samples with or without curcumin/turmeric were incubated overnight at 4 °C. The wells were washed twice with PBST. The wells were then blocked with 3% milk/PBST for 2 h at room temperature. The sera samples with or without curcumin/turmeric were diluted to required dilution (1:100 for human samples; 1:1000 for mice or rabbit samples) with diluent and each sample was added in quadruplicate at 100 µl/well. The ELISA was developed as described previously [10].

2.6. Staining of proteins by curcumin on SDS-PAGE

Unstained molecular weight standards, bovine serum albumin, HeLa cell antigens, La and Ro 60 autoantigens were resolved by SDS PAGE, fixed with 25% methanol and 10% acetic acid for 10 min, rinsed with water and stained with heat-solubilized curcumin for 30 minutes. The curcumin-stained gel was visualized by ultraviolet light using an UVP BioDoc-It™ system.

2.7. Surface Plasmon Resonance (SPR)

The BIAcore system was used to measure SPR. This system involves a sensor, in contact with a microfluidic cartridge. Ro MAP 273 was immobilized to a carboxy-methylated dextran matrix attached to the sensor surface. The other surface of the sensor, coated with a thin gold film faces the optical system. The SPR detector responds to refractive index changes in the vicinity of sensor surface as the Ro MAP 273 interacts with its ligand in fluid phase. The sensor surface was prepared essentially as reported earlier [10,11]. A mock surface was also prepared similarly, but without the Ro MAP 273, to check the binding of curcumin to the matrix. Typically, a single ligand surface was used for several analyses. Curcumin, prepared at 5 mg/ml (heated), was analyzed over the sensor surface to check for binding to Ro MAP.

Results and Discussion

In this study we used sera of patients with primary SS or SLE, sera from a Ro 273 peptide induced animal model of SS [14] and a commercial polyclonal anti-spectrin antibody in order to assess the ability of curcumin or turmeric to inhibit binding of these antibodies to

their respective antigens namely Ro 60, Ro 273 multiple antigenic peptide (MAP) or human spectrin.

Anti-Ro 60 antibodies from SS (Figure 1) bound significantly to Ro 60 while the controls did not. Similar results were obtained with sera for SLE patients (data not shown). Ro 60 binding by anti-Ro 60 positive SS sera was inhibited 24-43% ($35 \pm 5.8\%$; $n=9$) by heat-solubilized curcumin (Figure 1A). However, when heat-solubilized turmeric was used the binding was inhibited up to 74% ($58\% \pm 13.5$; $n=9$) (Figure 1B). Similarly Ro 60 binding by anti-Ro 60 positive SLE sera was inhibited by curcumin or TU by 36-52% (43 ± 6.6 ; $n=4$) or 61-70% (65 ± 4 ; $n=4$) respectively (data not shown).

Sera from a SS mouse model [14], obtained by immunizing with a Ro 60 peptide (Ro 273-289) bound significantly to the Ro MAP 273 while sera from Freund's immunized mice did not bind. Curcumin inhibited binding of anti-Ro 273 antibodies to Ro MAP 273 by 35-50% ($46\% \pm 8.9$; $n=5$) (Figure 2A) while TU inhibited by 55 to 60% ($58\% \pm 2.7$; $n=5$) (Figure 2B). The inhibition of binding to Ro 60 or Ro 273 MAP respectively by curcumin or TU was highly significant (up to $p<0.0000001$) (Figures 1 and 2).

In order to determine the protein binding specificity of curcumin, we stained molecular weight standards (purchased as unstained protein molecular weight standards), bovine serum albumin, La and Ro 60 autoantigens separated by SDS-PAGE, with heat-solubilized curcumin. Curcumin bound non-specifically to an array of 15 different proteins derived from a set of unstained molecular weight standards when each individual protein amounts was 500 ng or more. However, these proteins could also be stained with Coomassie Brilliant Blue, even at levels as low as 100 ng. Two hundred ng of Ro 60 or recombinant La autoantigen was not stained by curcumin, while it could be stained with CBB. Thus, there was no preferential staining of proteins with curcumin and the staining sensitivity was 4 to 5-fold less compared to that seen with Coomassie (Figure 3). To extend this information, we also determined the binding of heat-solubilized curcumin to MAP by surface plasmon resonance. The result of this study showed that curcumin bound to the Ro MAP 273 (Figure 4).

Since curcumin binds to proteins non-specifically, we hypothesized that it would inhibit other antibody antigen interactions as well and that it will not be restricted to autoimmune specificities. Purified human spectrin was used as the solid phase antigen and binding of anti-spectrin to spectrin in the presence and absence of curcumin or turmeric was determined by ELISA. Rabbit anti-human spectrin antibodies bound to spectrin with an OD of 2.57 while the rabbit control antibody did not bind. Binding of anti-spectrin to spectrin was inhibited 50% by curcumin and by 56% by turmeric. Control antibody did not bind to spectrin with or without curcumin/turmeric (Figure 5).

The most important limitation in using curcumin for *in vitro* studies or therapeutic purposes is its insolubility in water and consequently its poor bioavailability. Investigators have demonstrated [5] no detectable curcumin or curcumin metabolites in the blood or urine after patients with advanced colorectal cancer were administered 440 to 2200 mg of curcuma extract per day (36 to 180 mg of curcumin) for up to 29 days. Others have [6] shown that the peak concentration of curcumin in the serum following administration of 4, 6 and 8 g of curcumin were 0.51, 0.64 and 1.77 μM respectively. These authors also found that doses below 4 mg were barely detectable. Lao *et al.* [15] report finding no curcumin in the serum of volunteers given 0.5, 1.0, 2.0, 4.0, 6.0 or 8.0 g curcumin. However, these authors found that curcumin levels reached 50.5 and 51.2 ng/ml sera by four hours in two subjects administered 10 and 12 g of curcumin respectively. Yet another study [9] showed that only about 22-41 ng/ml were detectable in plasma even when 8 g curcumin/day was given orally.

Consequently, any method that aims to improve curcumin's solubility in water would be immensely useful to investigators attempting to find therapeutic advances to several debilitating and terminal illnesses.

We have shown a 12-fold increase in solubility of curcumin and a 3-fold increase in the solubility of turmeric by boiling a solution of curcumin/turmeric in water for 10 minutes. Profiling of the heat-extracted curcumin with matrix assisted laser desorption ionization mass spectrometry and spectrophotometry (400-700 nm) indicated no heat-mediated disintegration of curcumin [13,16]. By using an enzyme-linked immunosorbent assay that involved 4-hydroxy-2-nonenal (HNE) modification of a solid-phase antigen substrate [17], the heat-solubilized curcumin was found to inhibit HNE-protein modification by 80%. We have also shown that curcumin solubilized with mild alkali inhibited HNE-protein modification significantly [18]. Thus, inhibition of HNE modification may be a mechanism by which curcumin exerts its effect in many disorders [13,18].

We believe that one solution to this bioavailability problem would be to increase the solubility of curcumin before oral administration to patients. Thus, heat-solubilized curcumin should be considered in clinical trials involving curcumin since curcumin's full pharmacological potential is limited owing to its extremely limited water-solubility [19].

An earlier study showed that 90% of curcumin dissolved in 0.1 M phosphate buffer (pH 7.2) was broken down in 30 min [20]. We stored the heat solubilized curcumin or turmeric at 4 °C for 12 h or 72 h and measured the optical density at 405 nm as described earlier [13] following centrifugation at 16,000 g [13]. The level of heat-solubilized curcumin was found to decrease only 47% in 12 h and 67% in 72 h. However, there was only a 17% and 25% decrease in the corresponding turmeric samples.

Barik et al. [21] showed that curcumin binds very strongly to human serum albumin, with binding constants in the order of 10^4 - 10^5 M⁻¹, thus raising the possibility that it could be used as a carrier for curcumin *in vivo*. Recently Liu et al [22] demonstrated that curcumin binds to the CDRs of Fab of intravenous Ig, with binding constants of only 10^6 - 10^8 M⁻¹ raising the possibility that intravenous Ig could also serve to transport curcumin. Since albumin binds stronger to curcumin compared to intravenous Ig there is a competition for binding between albumin and intravenous Ig.

Studies in humans and animal models have shown that curcumin ameliorates autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, psoriasis, and inflammatory bowel disease by regulating inflammatory cytokines such as IL-1beta, IL-6, IL-12, TNF-alpha and IFN-gamma and associated JAK-STAT, AP-1, and NF-kappaB signaling pathways in immune cells [7]. Our results show that inhibition of antibody antigen interaction could be another mechanism for ameliorating autoimmune disorders, along with curcumin's ability to inhibit oxidative modification of proteins.

Oxidative damage and oxidative modification of proteins have been implicated in SLE and other autoimmune disorders [23,24]. By binding to proteins curcumin might prevent the oxidative modification of proteins induced by oxidative stress. Curcumin solubilized by heat in water [13,16,19, 25-27] is a plausible reagent for use in both *in vitro* and *in vivo* experiments as opposed to the use of curcumin solubilized in dimethylsulfoxide [28] the most commonly used solubilizing agent.

Investigators have recently used curcumin solubilized in DMSO (final concentration of 0.1%) to show that it could attenuate the toxicity of acrylamide on HepG2 cells [29]. DMSO has been used as a solvent for chemotherapeutic drugs and it has been used to treat rheumatic, pulmonary, gastrointestinal, neurological, urinary and dermatological disorders

owing to its anti-inflammatory properties [30]. The effects of DMSO on the outcomes of such studies are not completely clear yet. The DMSO levels reported to be safe varies considerably. Adverse effects of DMSO on the neuronal system have been reported. DMSO has been shown to induce apoptosis in a widespread manner in developing mouse cells at all ages tested, as well as induce neuronal loss at 0.5 and 1% [31]. Investigators have shown that DMSO accumulated in brain and was found to increase the metabolic rate. There is no practical concentration of DMSO that can be used in metabolic experiments without effect [32]. Our approach of heat solubilizing curcumin in water appears to be a simple and safe solution for overcoming the solubility problem associated with this polyphenol.

We obtained higher inhibition with turmeric extract than with the purified curcumin suggesting that some curcuminoid is lost in the purification process. Heat-solubilized curcumin/turmeric could prove useful as a therapeutic intervention in SS or SLE to suppress autoantibody/antigen interaction, to inhibit oxidative damage and thus reduce severity of disease manifestation. In addition, heat solubilized curcumin/turmeric could be used to stain proteins on SDS-PAGE gels as an alternative to heat-mediated staining of proteins with Coomassie [33]. The advantage here is that curcumin is non-toxic, even though it is less sensitive than Coomassie staining.

There are some limitations to this study. First, the results reported here are based on *in vitro* experiments. Plans are afoot to study the *in vivo* effects of heat-solubilized curcumin/turmeric in our experimental animal model of SS and possibly in spontaneous animal models of SLE. This will enable us to determine if the administration of heat-solubilized curcumin would increase bioavailability. If heat-solubilized curcumin does increase bioavailability it would be of interest to see if it would suppress autoimmunity along the line seen with induction of tolerance with oral feeding of Ro 60 autoantigen in experimental SS [34].

Second, the implications of the more or less promiscuous binding of curcumin by proteins may extend beyond the interference of antibody-antigen interactions, protein gel staining or intravenous Ig. It is possible that this could affect antibody interactions to non-self antigens, clearance of pathogens by antibodies, response to vaccination, or response to therapeutic antibodies employed in cancer or autoimmune diseases. The encouraging aspect of studies with non-heat solubilized curcumin thus far (up to 12 g/curcumin/day in some studies) is that most studies show highly significant beneficial effects [5-9, 13,15, 18, 20, 22, 35, 36] without any significant adverse effects. We hypothesize that heat-solubilized curcumin/turmeric would behave in this manner as well without significant adverse side effects as opposed to using DMSO.

Since Ro 60 autoantigen, is a major target of autoantibodies in patients suffering from rheumatic diseases [4,10,11,23], we hypothesize that the use of heat-solubilized turmeric/curcumin (especially in times of flare in disease) may be a better therapeutic approach compared to non-heat solubilized TU/CU (curcumin is practically insoluble in water maintained at room temperature) to ameliorate these diseases. The results of this study shows that heated curcumin retains its ability to bind autoantigens. In addition, it will be uncomplicated to administer specific quantities of heat-solubilized curcumin/turmeric in cooked food (especially omelette, since albumin binds curcumin strongly) or in infusions (after cooling and centrifugation or filtration to remove insoluble curcumin) to patients with autoimmune diseases.

Acknowledgments

Supported by NIH grants ARO49743, ARO48940 to RHS. We acknowledge the excellent assistance from Ms. Beverly Hurt of the Graphics Resources Center, OMRF for preparing Figure 2 of this manuscript.

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Figure 1.

Binding of sera from SS patients containing anti-Ro 60 antibody to Ro 60 autoantigen by ELISA. ELISA was carried out as mentioned in Materials and methods. Panel A- Binding of sera from SS patients to solid phase Ro 60 antigen and inhibition of binding to the antigen by heat-solubilized curcumin. Control refers to normal human sera. Panel B- Binding of sera from SS patients to solid phase Ro 60 antigen and inhibition of binding to the antigen by heat-solubilized turmeric. Control refers to normal human sera.

Values are means $\pm$ standard deviation for four determinations for each sample.

^a $p < 0.002$; ^b $p < 0.0001$; ^c $p < 0.001$; ^d $p < 0.000001$; ^e $p < 0.0001$; ^f $p < 0.0001$; ^g $p < 0.0001$; ^h $p < 0.0000001$; ⁱ $p < 0.0000001$; ^j $p < 0.00015$; ^k $p < 0.0001$; ^l $p < 0.00001$;

Figure 2.

Binding of sera containing anti-Ro 273 peptide antibody from an animal model of SS to Ro 273 multiple antigenic peptide (MAP) by ELISA. ELISA was carried out as mentioned in Materials and methods. Panel A- Binding of sera from mice immunized with Ro 273 peptide or Freund's adjuvant to solid phase Ro 273 MAP antigen and inhibition of binding to the antigen by heat-solubilized curcumin. Panel B- Binding of sera from mice immunized with Ro 273 peptide or Freund' adjuvant to solid phase Ro 273 MAP antigen and inhibition of binding to the antigen by heat-solubilized turmeric.

Values are means $\pm$ standard deviation for four determinations for each sample.

^m $p < 0.0004$; ⁿ $p < 0.00001$; ^o $p < 0.00001$; ^p $p < 0.0001$; ^q $p < 0.0003$; ^r $p < 0.00001$; ^s $p < 0.000001$; ^t $p < 0.00001$; ^u $p < 0.000012$; ^v $p < 0.00001$

Figure 3. SDS PAGE analysis of proteins stained by CU and CBB. Panel A: Lane 1-BSA; lane 2- Ro 273 MAP; lane 3-La; lane 4-Ro 60; lane 5- unstained molecular weight standards stained with CU. Panel B: Lane 6- unstained molecular weight standards (20 μ l) stained with CU; lane 7- unstained molecular weight standards (10 μ l) stained with CU; lane 8- unstained molecular weight standards (5 μ l) stained with CU; lane 9-unstained molecular weight standards (2.5 μ l) stained with CU. Panel C: Same as panel A but stained with CBB. Panel D: Same as panel B but stained with CBB.

Figure 4.

Surface plasmon resonance studies of curcumin binding to Ro MAP 273. Top-Curcumin analyzed over a mock sensor surface. Bottom-Curcumin analyzed over Ro MAP 273 coupled to the sensor surface. The dip in the sensorgram seen as curcumin passes over the sensor surface is owing to the differential refractive index of the heat-solubilized curcumin compared to the PBST used as the running buffer.

Figure 5.

Antispectrin antibodies binding to solid phase human spectrin in the presence or absence of curcumin (CU) or turmeric (TU). Samples used are given on the X-axis while optical density (OD) is given on the Y-axis. Sample 1 - antispectrin antibodies binding to spectrin in the absence of CU or TU; Sample 2- Control antibody binding to spectrin; Sample 3 - Antispectrin binding to spectrin in the presence of CU; Sample 4 - Antispectrin binding to spectrin in the presence of TU; Sample 5 - Control antibody binding to spectrin in the presence of CU; Sample 6 - Control antibody binding to spectrin in the presence of TU.

* p=0.0043; ** p=0.0025