

Fluorescent Labeling of Disulfide Proteins on 2D Gel for Screening Allergens: A Preliminary Study

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An experimental protocol was established to detect disulfide proteins as fluorescent spots on 2D gel. In summary, free sulfhydryl groups in a protein mixture were capped with nonfluorescent iodoacetamide, followed by chemical reduction of disulfide bonds, and labeling of newly exposed sulfhydryl groups with the fluorescent probe, monobromobimane. Disulfide proteins were detectable as fluorescent spots under the UV lamp. As accumulating evidence suggests that disulfide bonds are responsible for the allergenicity of many proteins, we sought to use this protocol to find new allergens. In a model experiment using soybean trypsin inhibitor, a well-known allergen with disulfide bonds, and myoglobin, which has a free sulfhydryl group, only the former was labeled with fluorescence. Application of the protocol to mite and pollen extracts facilitated detection of known allergens or putative allergens exhibiting sequence similarities to known allergens. In this note, we report the protocol as a complementary tool for screening allergens, which is now solely dependent on immunological recognition.

Current studies on comprehensive detection of allergens rely on the two-dimensional (2D)-PAGE of allergenic fractions, followed by immunoblot of the gels with patients' sera. The technique has been applied to wheat,¹ mite,² and pollen³ allergens. However, as reactivity to allergens varies among patients and the loss of reactivity or potency of allergens might occur during the denaturing and blotting procedures, some allergens may not have been detectable by conventional methods. Thus, a complementary screening method should work as a useful tool to find allergens that escaped the notice of the conventional method.

Accumulating evidence suggests that disulfide bonds are responsible for the allergenicity of some proteins.⁴ Using canine models, Buchanan and colleagues reported that reduction of disulfide bonds of wheat⁵ and milk⁶ allergens made them less allergenic. Sen et al.⁷ reported that formation of disulfide bonds

rendered Ara h2, a major peanut allergen, resistant to proteases of the digestive system. They also reported on the presence of IgE-binding epitopes in recalcitrant peptide fragments of Ara h2 that survived in vitro digestion by gastrointestinal proteases. By a comprehensive analysis based on disulfide proteome,⁸ we have shown that allergenic fractions of peanut⁹ and rice¹⁰ are mainly composed of disulfide proteins. So detection of disulfide proteins may facilitate screening of possible allergens.

We sought to selectively label with fluorescence the disulfide proteins in crude extracts of mites and pollen. First, the proteins' free sulfhydryl groups (SHs) were capped with nonfluorescent iodoacetamide (IAA). Then, proteins were treated with dithiothreitol (DTT) to expose SHs taking part in the disulfide bonds. Finally, they were labeled by fluorescent probe, monobromobimane (mBBR). Known allergens and putative allergens that shared sequence similarities with known allergens were successfully detected by the protocol.

MATERIALS AND METHODS

Mites were purchased from Osaka Pharmaceuticals Co, Ltd. (Osaka, Japan). Ragweed pollens were from Sigma (St. Louis, MO). Monobromobimane was from Calbiochem (San Diego, CA). Bio-Lytes was from Bio-Rad (Hercules, CA). Other chemicals and biochemicals were purchased from commercial sources and were of the highest quality available.

Preparation of Crude Protein Extracts. Whole mite culture or ragweed pollen was defatted with diethyl ether and ground to a fine powder with mortar and pestle. Proteins were extracted with 0.1 M Tris-HCl buffer (pH 8.0) in the presence of 1 mM EDTA and PMSF, followed by centrifugation at 14000g for 30 min. The supernatant was filtered through a centrifugal filter Ultrafree CL (Millipore, Bedford, MA) and the filtrate was desalted with a centrifugal filter Microcon YM-10 (Millipore); both operations were performed according to manufacturers' instructions. The concentrate was dissolved into a DTT-free lysis buffer, composed of 0.5% (3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS), 8 M urea, 0.1% Bio-Lytes, and 0.001% Bromophenol Blue. The solution was then subjected to fluorescent labeling of disulfide proteins.

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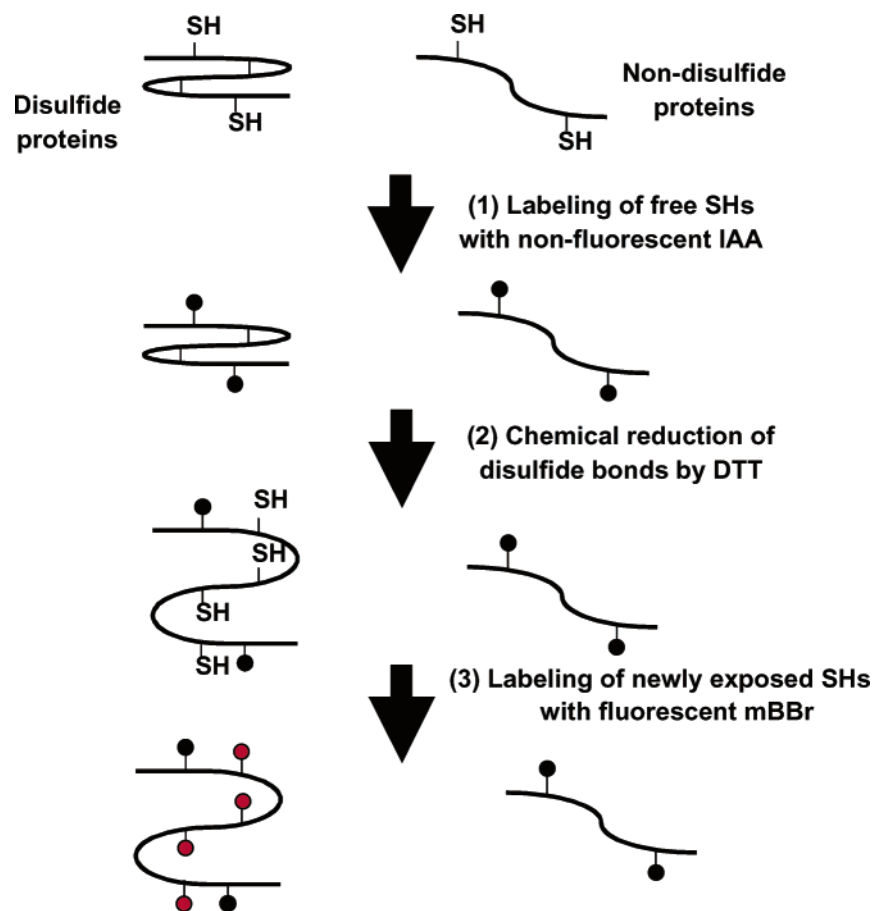


Figure 1. Protocol to specifically label disulfide proteins: black filled dot, sulfhydryl groups capped by IAA; red filled dot, sulfhydryl groups fluorescently labeled by mBBR.

Fluorescent Labeling of Disulfide Proteins. Iodoacetamide was added to a protein extract to a final concentration of 5 mM and was incubated for 1 h at room temperature. Then, DTT was added to a final concentration of 5 mM, and the mixture was stored at room temperature for 1 h. Finally, mBBR was added to a final concentration of 10 mM. The solution was stored for 15 min and ready for a separation on the 2D-PAGE.

Isoelectric Focusing/SDS 2D-PAGE. An immobilized pH gradient gel (IPG) strip (pH 3–10) was swollen by a protein sample, and IEF was performed using the Protean IEF Cell (Bio-Rad) according to the manufacturer's instructions. After that, the IPG strip was immersed in a Laemmli sample buffer,¹¹ followed by 10–20% acrylamide gradient SDS–PAGE. The resultant gel was stored in 30% methanol/5% acetic acid solution and was examined under an FAS-2525 365-nm UV light (Toyobo, Tokyo, Japan) to detect fluorescent spots. Then, the gel was incubated overnight at room temperature in 30% methanol/5% acetic acid containing 0.05% Coomassie Blue R-250 (CBB) and was destained by 30% methanol/5% acetic acid until protein spots were visible.

Identification (Amino Acid Sequence) of Disulfide Proteins. In the case of mite and pollen extracts, each fluorescent spot was cut out from the 2D gel without CBB staining. In-gel digestion by trypsin was performed as described by Rosenfeld et al.¹² The N-terminal sequences of the fractionated tryptic peptides

were determined by a Procise 494 HT protein sequencer (Applied Biosystems, Foster City, CA).

RESULTS AND DISCUSSION

Figure 1 shows the protocol for specific labeling of disulfide-forming SHs in a mixture of proteins. As free SHs are capped in advance by nonfluorescent IAA, successive exposure of disulfide-forming SHs by DTT and treatment by mBBR selectively label disulfide proteins with fluorescence.

To evaluate the strategy, a model experiment was performed. Soybean trypsin inhibitor (STI) has two disulfide bonds¹³ and also is well known as an allergen.¹⁴ Myoglobin from human skeletal muscle has one free SH group and does not contain any disulfide bond.¹⁵ Fifty picomoles of STI and 250 pmol of myoglobin were mixed, and the mixture was subjected to IAA, DTT, and mBBR in the order shown in Figure 1. If both free and disulfide-forming SHs are labeled, the intensity of fluorescence should be same between STI and myoglobin. As shown in Figure 2B, only STI was labeled with fluorescence. When the same gel was treated with CBB, both proteins were stained (Figure 2A). Thus, we demonstrated that the free SH of the myoglobin was capped by nonfluorescent IAA and only the disulfide-forming SHs of STI were labeled with fluorescent mBBR. The data suggest that the protocol worked successfully to selectively label disulfide protein with fluorescence.

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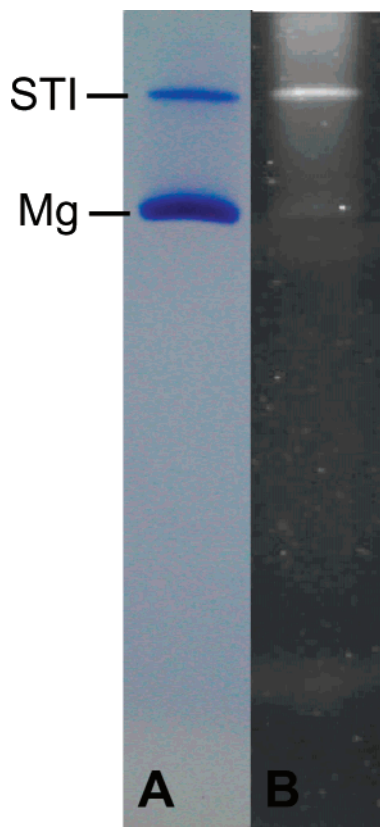


Figure 2. SDS-PAGE profile of soybean trypsin inhibitor (STI) and human skeletal myoglobin (Mg). Fifty picomoles of STI and 250 pmol of Mg were applied on the gel. (A) Coomassie staining of proteins; (B) fluorescent labeling of disulfide proteins.

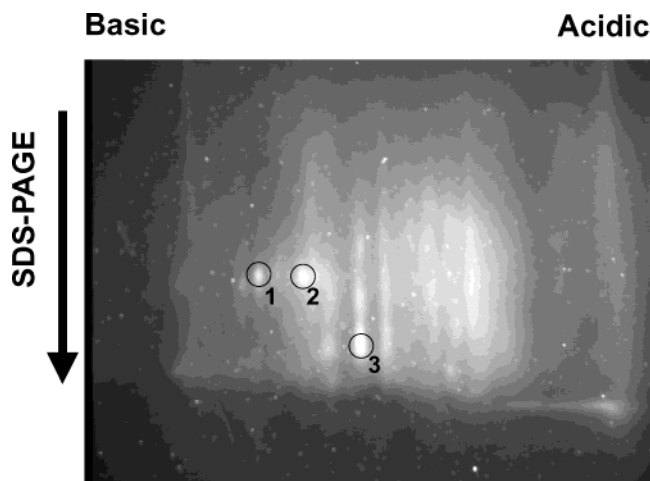


Figure 3. Detection of disulfide proteins of mite extract on 2D-PAGE. One hundred micrograms of total protein was applied on the gel.

Next, the protocol was applied to a crude extract of mites. Three major fluorescent spots were clearly visible under the UV light (Figure 3). These spots were subjected to internal amino acid sequence analyses, and the amino acid sequences were subjected to the SWISS-PROT and TrEMBL databases using the FASTA algorithm (Table 1). Disulfide proteins 1 and 2 were identified as the major mite allergen Der P2.¹⁶ Protein 3 was

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Table 1. Internal Amino Acid Sequence Analysis of Disulfide Proteins in Crude Extracts from Mite and Pollen

no.	internal sequence	homologous protein	matches (ref)
mite			
1	YTWNVPK	mite allergen DER P2	7/7 (16)
2	GKPFQLEAVFEA	mite allergen DER P2	12/12 (16)
3	FIDCGHNEVK	mite allergen GLY D2	8/10 (17)
pollen			
1	CIEWEGAK	anthem-specific protein SF18	7/8 (18)
2	LCEKPSLTXS	cystein-rich anti-fungal protein	9/11 (19)
3	VDHIVGEEK	ABA-1 allergen	6/9 (21)
4	GDFPVFYVTK	unidentified	
5	QIAQGDELVFNY	unidentified	
6	QIVQGDELVFK	unidentified	

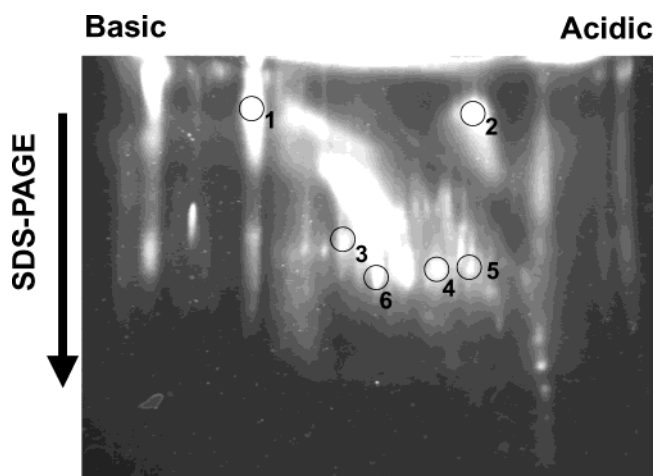


Figure 4. Detection of disulfide proteins of pollen extract on 2D-PAGE. One hundred micrograms of total protein was applied on the gel.

unidentified but exhibited a high sequence similarity to the major mite allergen Gly D2.¹⁷ One of the conveniences of our method is that the fluorescent spots can be applied to the following internal sequence analyses without cumbersome CBB staining and destaining procedures. Also, fluorescent labeling facilitates detection of allergen candidates expressed in small amounts.

Application to pollen crude extract also detected disulfide proteins (Figure 4). Disulfide proteins 1 and 2 exhibited a high sequence similarity to the major pollen allergen SF18¹⁸ and the cystein-rich antifungal protein,¹⁹ respectively (Table 1). Both belong to the allergenic defensin family.²⁰ Protein 3 exhibited sequence similarity to ABA-1,²¹ a major allergen of parasite worms. Proteins 4–6 did not exhibit significant sequence similarity to any known proteins.

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In the preliminary experiments performed here on mite and pollen extracts, most of the detected disulfide proteins (6 out of 9) were identified as known major allergens or putative allergens exhibiting sequence similarities to known allergens. Also, unidentified proteins found in pollen extract (proteins no. 4–6) may be allergenic. Immunological studies are in progress in our laboratory to investigate the allergenicity of these suspected allergens.

Detection of disulfide proteins worked successfully as a “preliminary study” to screen allergens in the case of mite and pollen extract. However, there could be proteins that react with mBBR under the specified conditions that are not allergens. Further studies including application to other allergens and detection of allergens using other methods are in progress in our laboratory to evaluate the universal validity of this method.

CONCLUSION

Our strategy appeared a useful tool in detecting disulfide proteins on 2D gel. Although all disulfide proteins are not necessarily allergens, the application of this procedure on mite

and pollen extracts led to detection of several known or possible new allergens (Table 1). Also, fluorescent labeling should facilitate detection of allergen candidates expressed in small amounts. Our strategy may be a useful tool in finding new allergens that have escaped the conventional screening method now relying solely on immunological recognition.

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