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Stoichiometric Dye-Binding Procedure for the Determination of the Reactive Lysine Content of Soya Bean Protein

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ABSTRACT

A new analytical method has been developed for the determination of the reactive lysine content of soya bean protein. The method is based on the reaction of the free basic groups of the protein with 1-phenylazo-2 naphthol-6,8 disulphonic acid. With regard to the stoichiometry of the procedure, it has been proved, contrary to earlier reports, that the basic amino acids, histidine, arginine and lysine, each combine with one mole of the dye. After acylation with propionic anhydride lysine alone loses its dye reactivity. The usefulness of the proposed method has been demonstrated by the determination of the reactive lysine content of several untreated, heat-treated and acid-treated soya bean samples. The results show that heat damage of about 5% in reactive lysine content can be measured in 1·5 h with good reproducibility.

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INTRODUCTION

The most convenient methods of determining heat damage to proteins are dye-binding procedures because they: (i) are inexpensive; (ii) are not time- or labour-consuming and (iii) can be carried out without hydrolysing the protein. The last of these is the most important advantage; the results of a reaction at ambient temperature can be expected to best reflect the equilibrium state of proteins.

Attention was drawn to the chemical stoichiometric interaction between proteins and certain acid and basic dyes by Loeb (1924). Chapman *et al.* (1927) and Rawlins & Schmidt (1929; 1930) studied the interaction of proteins with acid and basic dyes as a function of pH. They corroborated the results of Loeb (1924)—(i) sulphonic acids, mainly azo-dyes, react with the free basic groups of proteins at pH 2 in the stoichiometric molar ratio of $\sim 1 \cdot 1$; (ii) basic dyes combine with the free carboxy groups of proteins at pH 11, somewhat less reproducibly, but also in a nearly 1:1 molar ratio.

The interaction of the free basic groups (histidine, arginine and lysine; that is, basic amino acids (BAA)) of proteins was used partly for the determination of their total nitrogen content (Udy, 1956, 1971; Pomeranz, 1965; Sherbon, 1967; Hymowitz *et al.*, 1969; Khan, 1978; Goh & Clandinin, 1978; Goh *et al.*, 1979) and partly for following the qualitative change of the total nitrogen content by means of Maillard reactions (Hurrell & Carpenter, 1975; Hurrell *et al.*, 1979; Walker, 1979*a*, *b*). In earlier work, Orange G, i.e. 1-phenylazo-2-naphthol-6,8-disulphonic acid (OG) and, after the publication of Udy's work in 1971, Acid Orange 12, i.e. 1-phenylazo-2-naphthol-6-monosulphonic acid (AO-12) were preferably used. Udy (1971) reported that the colour sensitivity of OG is half of that of AO-12. He based his assumption on the difference in structure of the two molecules. He supposed that, in the case of OG (a disulphonic acid), two amino groups can be bound to one azo-chromophore whereas AO-12 (a monosulphonic acid) can combine only with one amino group. He did not prove his assumption by experimental data; neither could he support it from the literature. In earlier experiments with OG–protein reactions, molar ratios of dye to protein were used (dye to protein ratio was always < 1) at which no actual stoichiometry could be studied (Table 1).

Further exhaustive investigations concerning the determination of reactive lysine, based on its direct dye-binding capacity (Hurrell & Carpenter, 1975; Hurrell *et al.*, 1979) were carried out with AO-12.

TABLE 1
Reaction Conditions and Results of Dye-binding Capacity Measurements of Soya Bean Proteins with AO-12 and OG
(Literature data calculated by the present authors)

Dye	*Dye:BAA ratio (M/M)*[a]		*Reference*
	Reacted	*Bound*	
OG	0·20	0·11	Udy (1956)
	0·48	0·34	Moran *et al.* (1963)
	0·52	0·33	Goh & Clandinin (1978)
	0·79	0·38	Goh & Clandinin (1978)
AO-12	3·05–1·40	1·09–1·04	Udy (1971)
	2·16–1·73	0·97	Hurrell & Carpenter (1979)
	No data available	1·00	Hurrell & Carpenter (1979)
	1·26	0·85	Goh & Clandinin (1978)
	1·56	1·03[b]	Hurrell *et al.* (1979)
		1·04[c]	Hurrell *et al.* (1979)

[a] The total amounts of protein BAA, expressed in moles (M), were calculated according to the data of Kellor (1974). The latter results were in good agreement with the BAA content of our model sample 1, determined as trifluoracetyl *n*-butyl esters.

These data are as follows: 1 g of protein contains 6·25 w/w%, i.e. $4{\cdot}27 \times 10^{-4}$ M lysine, 7·06 w/w%, i.e. $4{\cdot}36 \times 10^{-4}$ M arginine and 2·84 w/w%, i.e. $1{\cdot}83 \times 10^{-4}$ M histidine or 16·15 w/w%, i.e. $10{\cdot}46 \times 10^{-4}$ M BAA, in total.

[b] Soya bean (Glycine max.).

[c] Soya flour, Z78.

In the present work, the stoichiometry in the course of a reaction between soya bean protein and OG is clarified. The reactive lysine content, before and after propionylation, is calculated from the difference in the dye-binding capacity for a certain dye, as proposed by Hurrell & Carpenter (1979). The performance of their procedure is demonstrated by the dye-binding data of some soya bean proteins pretreated (denaturized) in different ways in order to make them useful in human and animal nutrition.

EXPERIMENTAL

Model materials

Samples 1, 2, 3 and 4 comprised untreated defatted (sample 1) and fat-containing (samples 2, 3 and 4) soya beans. They were of different

commercial origin but the same variety—NKS 1346 (Glycine max.). Samples, denoted below by the numbers 1–3 following the original sample number, are the products of the untreated samples processed by means of various procedures: samples 1/1 and 4/1 were heated to 100 °C for 5 min, applying microwave heat treatment. Sample 1/2 was acidified to pH 2 with hydrochloric acid and was not neutralized. Samples 2/1, 2/2 and 3/1 were acidified to pH 2 with a mixture of phosphoric and hydrochloric acids (mole ratio, 2:1) and neutralized immediately with sodium carbonate and calcium carbonate.

Reagents

Propionic anhydride, anhydrous sodium acetate, OG (Acid Orange 10; CI 16230) and the components of the standard dye solution were all analytical grade chemicals and products of Reanal (Hungary). Glass filter paper was Grade GF/A Whatman (Great Britain). The OG standard dye solution contained 9 g of OG (weighed with analytical accuracy), 300 cm^3 of glacial acetic acid, 100 g of oxalic acid dihydrate, 17 g of potassium dihydrogen phosphate and water to 5·0 litres. It can be stored at room temperature for several months.

Apparatus

Electrical shaker: Vibroterm with twenty-four places (Labor MIM, Hungary). Spectrophotometer: Spectromom 361 (MOM, Hungary).

Determination of the total dye-binding capacity (TDBC)

Fifteen milligrams of soya bean (ground to 60 mesh, weighed with analytical accuracy) were transferred to a 100 cm^3 glass stoppered flask; three 6 mm glass beads and 2 cm^3 2·0M sodium acetate were added and the stoppered flask was shaken for 10 min. Next, 40 cm^3 of standard dye solution was pipetted into the flask and shaken for 1 h. It was then filtered through a filter cap filled with fibre glass filter paper and a 1 cm^3 aliquot of the filtrate was diluted 1:100 in a volumetric flask with distilled water. The optical density of the diluted filtrate was determined at 470 nm against water. The extinction was compared directly with that of a standard dye solution diluted to the same extent and containing all of the reagent used for the TDBC determinations.

Determination of the dye-binding capacity after propionylation (DBCAP)

The TDBC procedure was followed, with the following exception: before the reaction with the standard dye solution, 0·4 cm^3 of propionic anhydride was transferred to the sample previously reacted for 10 min with the sodium acetate solution and shaken for 15 min. Next, 40 cm^3 of standard dye solution was added.

RESULTS AND DISCUSSION

Preliminary experiments have shown that the optimum dye to soya bean protein ratio should be in the range 2·3–3·5. Therefore, optimization of time for propionylation and dye-binding has been made at a dye: protein molar ratio of 2·3 (Table 2). It is apparent from our investigations that acylation is complete in 10 min, whereas, for dye-binding, 45 min are needed. Consequently, we allow 15 min for propionylation and 60 min for the reaction with OG. In order to obtain better reproducibility, the samples were shaken with sodium acetate solution before mixing them with the dye and propionic anhydride solution in the presence of glass

TABLE 2

Model Investigations of Reaction Time Required for Quantitative Dye-binding[a] and Propionylation

Dye-binding time (min)	*Bound OG*			*Propionylation[b] time (min)*	*Bound OG*		
	(mg per gram of protein)		*%[c]*		*(mg per gram of protein)*		*%[c]*
10	357		73·5	0	397		44·7
30	410		84·4	5	304		85·4
45	485		100	10	287		100
60	489	486[d]	100	15	288	287[d]	100
90	488		100	30	286		100
120	482		100	60	287		100

[a] All values are the average of triplicate determinations, carried out with 150 mg of sample 1.

[b] Followed by 1 hour of dye binding.

[c] Expressed in per cent of the quantitative reactions, i.e. TDBC = 486 mg OG per gram of protein. DBCAP = 486 − 287 = 199 mg per gram of protein.

[d] Average value of bound OG.

beads for 10 min. This intimate wetting with sodium acetate resulted in (i) reproducible binding values and (ii) higher TDBC and DBCAP which were 4–5% closer to the theoretical values.

In order to clarify the stoichiometry of the OG–soya bean protein reaction, identical amounts of dyes, ~70–75 mg, were reacted with different amounts of proteins containing various BAA's (Table 3). The stoichiometry of the dye-binding reaction and its reproducibility were measured at the same time for untreated (sample 1), heat treated (sample 1/1) and acid treated (sample 1/2) soya bean proteins. It has been proved that, independently of the history of the samples: (i) OG combines with the basic amino acids of the protein in a molar ratio of approximately 1:1 and (ii) the reproducibility of the method is satisfactory.

Optimum dye–protein ratios to be used in dye-binding reactions, TDBC and DBCAP values are those which best approximate the theoretical values, i.e. 1 mole of dye to 1 mole of BAA or 1 mole of dye to 1 mole of lysine. Thus, the molar ratio due to protein of 2·3–2·5 is regarded as an optimum range. Further studies have proved that this is also valid for the heat- and acid-treated samples.

The reproducibility of the suggested procedure has been demonstrated on three further untreated samples with different protein contents by means of their TDBC and DBCAP values (Table 4). Considering the protein and amino acid contents of these samples, it is seen that the optimum dye to BAA molar ratio lies between 2·3 and 3·5, similarly to sample 1.

The usefulness of our method in the determination of the reactive lysine content, in addition to sample 1, is demonstrated by measurements carried out on the products of samples 2–4 (Table 5). As can be seen, the reactive lysine content of the products from samples 2 and 3 agrees with that measured in the original sample. The decrease in the reactive lysine content of sample 4/1 relative to that of sample 4 is in accordance with the treatment. In parallel, each sample was tested by feeding to chickens and pigs. In agreement with our results it has been proved that the nutritional value of samples 2/1, 2/2 and 3/1, with unchanged reactive lysine, corresponds with calculated data whilst the heat treated and reduced lysine-content samples (1/1, 1/2 and 4/1) have less biological value.

Our method is suitable for measuring the reactive lysine content of a protein without it being hydrolysed: 23·2% and 19·8% decreases in the reactive lysine content have been measured for the heat treated samples 1/2 and 4/2, respectively (Tables 2 and 5) whereas, in sample 4/1, which

TABLE 3(a)

Stoichiometry of the TDBC and DBCAP of an Untreated Soya Bean (Sample 1) as a Function of the Interacted Dye–Protein Ratio

Sample	*Soya bean weight (mg)*											
	50			*100*			*150*			*200*		
	Interacted dye/BAA ratio (M/M)[a]											
	6·90			*3·45*			*2·30*			*1·72*		
	Bound OG (mg per gram of protein) *A values = TDBC* *B values = DBCAP*											
	A	*B*	*A − B*	*A*	*B*	*A − B*	*A*	*B*	*A − B*	*A*	*B*	*A − B*
1												
a	564	342	222	493	331	162	482	283	199	426	276	150
b	556	342	214	489	332	157	488	294	194	432	274	158
c	540	348	192	523	298	225	487	293	194	432	277	155
d	540	351	189	509	301	208	497	294	200	433	279	254
e				509	298	211	482	291	191	433	272	161
f				501	313	188	480	292	188	434	274	160
$\bar{x}$	550 (1·16)	346	204 (1·06)	504 (1·07)	312	192 (0·99)	486 (1·03)	291	195 (1·01)	431 (0·91)	275	156 (0·81)
SD	9·1	4·5		12·3	27·7	27·8	5·2	4·2	5·3	2·97	2·52	4·14
SD%	1·7	1·3		2·5	8·9	14·5	1·1	1·4	2·7	0·7	0·9	2·7

[a] See Table 3(c).

TABLE 3(b)

Stoichiometry of the TDBC and DBCAP of Heat Treated Soya Bean (Sample 1/1) as a Function of the Interacted Dye–Protein Ratio

Sample	*Soya bean weight (mg)*								
	100			*150*			*200*		
	Interacted dye/BAA ratio (M/M)[a]								
	3·45			*2·30*			*1·72*		
	Bound OG (mg per gram of protein) *A values = TDBC* *B values = DBCAP*								
	A	*B*	*A − B*	*A*	*B*	*A − B*	*A*	*B*	*A − B*
1/1									
a	388	245	143	381	241	140	346	243	102
b	388	247	141	387	257	130	352	227	125
c	362	237	125	388	245	143	366	259	107
d	362	230	132	388	247	141	368	256	112
e	375	219	156	409	242	167	382	261	121
f	390	237	153	388	265	123	386	260	126
$\bar{x}$	378 (0·80)	236	142 (0·74)	390 (0·82)	249	141 (0·73)	367 (0·78)	251	116 (0·60)
SD	13·1	10·3	11·0	9·7	9·5	15·0	15·8	13·5	97·2
SD%	3·5	4·4	7·8	2·5	3·8	10·6	4·3	5·4	8·4

[a] See Table 3(c).

TABLE 3(c)

Stoichiometry of the TDBC and DBCAP of an Acid-Treated Soya Bean (Sample 1/2) as a Function of the Interacted Dye–Protein Ratio

Sample	*Soya bean weight (mg)*								
	100			*150*			*200*		
	Interacted dye/BAA ratio (M/M)[a]								
	3·45			*2·30*			*1·72*		
	Bound OG (mg per gram of protein) *A values = TDBC* *B values = DBCAP*								
	A	*B*	*A − B*	*A*	*B*	*A − B*	*A*	*B*	*A − B*
1/2									
a	479	303	176	448	283	165	414	264	150
b	485	320	165	443	283	160	404	259	145
c	479	320	159	431	273	158	425	265	140
d	484	320	164	419	274	145	426	265	141
$\bar{x}$	482 (1·02)	316	166 (0·86)	435 (0·92)	278	157 (0·81)	417 (0·88)	263	144 (0·75)
SD	3·2	8·5	7·2	13·0	5·5	7·3	10·4	2·9	4·5
SD%	0·7	2·7	4·3	3·0	2·0	4·6	2·5	1·1	3·2

[a] Calculated as detailed in Table 1. a-f = Parallel measurements. In parentheses: Bound Dye/BAA (M/M) in the cases of A values. Bound Dye/L (M/M) in the cases of A − B values.

Molecular weight of OG = 452. Protein content of sample 1 = 44·0% (N × 6·25). BAA content of sample 1 = $1{\cdot}046 \times 10^{-3}$ M per gram of protein = theoretical TDBC = 473 mg of dye per gram of protein. Lysine content of sample 1 = $4{\cdot}27 \times 10^{-3}$ M per gram of protein = theoretical DBCAP = 193 mg of dye per gram of protein.

$\bar{x}$ = Average. SD = Standard deviation.

TABLE 4
Stoichiometry of the TDBC and DBCAP of Soya Bean Samples 2–4 with Different Protein Contents

Sample	*Protein content* (%)[a]	*Soya bean weight (mg)*											
		50		*100*		*150*		*200*		*250*		*300*	
		Bound OG/BAA (M/M) = *I*								*OG/L* (M/M) = *II*			
		I	*II*	*I*	*II*	*I*	*II*	*I*	*II*	*I*	*II*	*I*	*II*
2	45·1	1·03	1·02	1·04	0·97	0·99	0·93	0·95	0·89				
3	46·4	1·00	1·03	1·03	0·98	1·00	0·90	0·93	0·78				
4	31·7			1·10	0·85	1·09	1·02	1·03	0·96	0·94	0·86	0·90	0·82

[a] Nx6·25. The amino acid distribution of samples 2–4 was the same as that of sample 1, within the experimental error of the gas–liquid chromatographic determinations. Detailed data in Table 2.

TABLE 5
Changes of Reactive Lysine Content, i.e. TDBC and DBCAP Values, in Samples 2–4 after Different Denaturation Processes[a]

Sample	*Bound OG (mg per gram of protein)*		
	A	*B*	*A – B*
2	468	289	179
2/1	476	297	179
2/2	479	308	171
3	473	299	174
3/1	476	300	176
4	487	302	185
4/1	446	296	150

[a] Weighed sample: 150 mg. A, B and A – B values as in Table 3.

was treated by a mineral acid and not neutralized, a 14·7 % decrease in the reactive lysine content was found.

According to our data, this method is a sensitive, and practically very important, indicator of minor decreases in reactive lysine content of proteins. It has numerous advantages over methods requiring previous hydrolysis of the protein. (i) It requires 4–24 h less time than procedures applicable only after hydrolysis which are also very tedious. (ii) Equilibrium relations of Maillard products are modified only insignificantly. (iii) Because of the last fact there is no need for empirical correction factors.

The general applicability of the stoichiometric OG–protein dye-binding procedure was proved with various proteins. The details of the stoichiometry of OG dye-binding and its comparison with that of AO-12, with regard to the amino acid composition of pure proteins (bovine plasma albumin, casein, γ-globulin and human serum albumin) and that of protein-containing food and feedstuffs (milk powder, fish, meat and bone meal) will be presented in a subsequent paper.

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