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Received for review May 11, 1987. Accepted January 26, 1988. This investigation was supported by Agriculture Canada in the form of a DSS Contract (No. OSU81-00523) with McGill University and by McGill University in the form of postgraduate bursary awarded to C.N.K. (1984–1985).

Levels of Formaldehyde in Milk, Blood, and Tissues of Dairy Cows and Calves Consuming Formalin-Treated Whey[1]

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Residual formaldehyde (CH_2O) in the tissues, blood, and milk of calves and cows fed whey preserved with various levels of formalin was measured by gas chromatography with an electron-capture detection (ECD) system after derivatization with (2,4-dinitrophenyl)hydrazine. The morning milk from cows fed whey preserved with 0.05, 0.10, and 0.15% formalin in three individual trials contained an average of 0.034, 0.095, and 0.208 mg of CH_2O kg^{-1}, respectively. Endogenous levels of CH_2O in milk from cows fed whey without added formalin were below the detection limit of 0.026 mg of CH_2O kg^{-1}. Average blood CH_2O concentration in cows fed 0.15% formalin-treated whey was greater ($P < 0.01$) than that of control cows at 33 days (0.831 ± 0.132 $mg \cdot kg^{-1}$ vs. 0.615 ± 0.110 $mg \cdot kg^{-1}$) but not significantly ($P > 0.05$) different 9 days (0.825 ± 0.061 $mg \cdot kg^{-1}$ vs 0.766 ± 0.045 $mg \cdot kg^{-1}$) after formalin treatment was initiated. Fresh muscle tissue from calves consuming whey containing 0.10% formalin had a significantly ($P < 0.05$) higher concentration of CH_2O (0.256 $mg \cdot kg^{-1}$) than fresh muscle tissue from control calves or calves consuming whey containing 0.05% formalin (0.178 and 0.206 $mg \cdot kg^{-1}$, respectively). There was no significant ($P > 0.05$) effect due to formalin preservation of whey on CH_2O concentrations in blood and frozen muscle, kidney, liver, and heart tissues.

Production of liquid whey from the manufacture of cheddar cheese (sweet whey) and cottage cheese (acid whey) exceeds the demand for whey products to be incorporated into animal and human foods. Without costly treatment prior to disposal, this excess energy-rich resource becomes a serious environmental pollutant. Ideally, all liquid whey could be fed directly to livestock, with benefits accruing to the dairy industry by eliminating current disposal costs. Unfortunately centers of livestock production are often not in close enough proximity to cheese plants to allow daily delivery of fresh whey at an attractive cost to producers. Whey is an unstable feedstuff and should be offered fresh to maintain palatability.

An alternative to daily whey delivery would be to add a preservative to the whey and store it in covered tanks. The addition of formalin (37% formaldehyde [CH_2O], w/w) at a level of 0.1% by weight inhibits lactose conversion in whey with no reported detrimental effects on health and growth of livestock (Modler et al., 1980). The metabolic fate of ingested CH_2O in livestock species is largely unknown, as is the effect of ingested CH_2O on concentration of CH_2O in vivo and the potential health risks to humans from chronic exposure to food products containing CH_2O. Interest by the dairy industry in obtaining government approval of formalin as a preservative for whey led to support for an investigation of the transfer of CH_2O from ingested formalin-treated whey to tissues and milk of dairy cattle.

The purpose of this investigation was to apply a new analytical technique to the determination of free and reversibly bound CH_2O in tissues and milk of dairy animals ingesting formalin-treated acid whey under conditions simulating a commercial operation. Although the chromotropic acid assay has been a popular method for analysis of CH_2O in livestock tissues and milk (Mills et al., 1972; Kreula and Rauramaa, 1976; Florence and Milner, 1981; Syrjälä-qvist and Setälä, 1982a,b), the sensitivity of this method has been considered inadequate for determination of endogenous CH_2O in animal tissues (Heck et al., 1982). Analysis of aldehydes as derivatives of (2,4-dinitrophenyl)hydrazine by gas chromatography with ^{63}Ni electron-capture detection [ECD] (Buckley et al., 1986) provides the sensitivity and specificity required to detect endogenous levels of CH_2O.

EXPERIMENTAL SECTION

Animals and Diets. *Lactating Cows.* The experiment involved the use of 12 Holstein dairy cows in their first trimester of lactation with a history of zero to eight previous lactations (group average of three lactations) and no previous exposure to CH_2O-treated feeds. The cows were trained to electronic gates (Calan Broadbent Doors, American Calan, Inc., Northwood, NH) to ensure that each animal received the designated treatment. The diet consisted of a low-enery pelleted concentrate (Table I) fed at a level of 1 kg of concentrate/3 kg of milk, liquid acid whey (Table II), and good-quality grass hay offered ad libitum.

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0021-8561/88/1436-1146$01.50/0 Published 1988 by the American Chemical Society

Table I. Composition of the Concentrate Fed to Cows with Liquid Acid Whey

ingredient, % of diet	
oats	68.00
soybean meal	14.60
molasses	3.95
canola meal	9.60
limestone	3.00
mineral–vitamin mix[a]	0.85
chem constituent, % of dry matter	
crude protein	21.00
acid detergent fiber	10.25
DE (Mcal)	3.9
calcium	1.36
phosphorus	0.46
magnesium	0.20
sodium chloride	0.15

[a] Provided the following per kilogram of diet: cobalt, 0.279 mg; copper, 1.760 g; manganese, 50 mg; zinc, 50 mg; iodine, 1.5 mg; selenium, 0.20 mg; vitamin A, 301 IU/kg DM; vitamin D, 25 IU/kg DM.

Table II. Estimated Chemical Composition of Whey on a Liquid (As Fed) Basis

acidity, pH	3.98
water	95.2
total solids	4.8
solids content, g	
lactose	3.2
protein	0.5
fat	<0.05
lactic acid	0.4
ash	0.5
gross energy, kcal/kg	4.1
ash content	
calcium	0.10
phosphorus	0.08
iron	0.001
potassium	0.14
sodium	0.05

A total of 49 days were allowed for the cows to adjust to the experimental conditions and to establish uniform maximum whey consumption.

The experiment was divided into three trials of 35 days each with 14-day intervals between trials. The six control cows were fed 75 kg·head^{-1}·day^{-1} of untreated whey in all three trials, while the remaining six were fed 75 kg·head^{-1}·day^{-1} of whey treated with 0.05%, 0.10%, and 0.15% formalin (0.0185%, 0.0370%, 0.0555% CH_2O), respectively. The calculated amounts of ingested CH_2O were 13.9 g·head^{-1}·day^{-1} in trial 1, 27.8 g·head^{-1}·day^{-1} in trial 2, and 41.6 g·head^{-1}·day^{-1} in trial 3. Whey was fed once a day at 1000 h. All whey was consumed by 1630 h.

Morning milk was sampled on days −3, 2, 3, 4, 5, 6, 13, 20, 27, and 34 of each 35-day trial period, with sampling taking place 22 h after the cows were last fed whey. Aliquots of milk were obtained by bubbling air briefly through the collection jar containing the morning milk of each animal, thereby mixing the milk. A 250-mL aliquot was then drained off into a sample container. Milk was also sampled 46 h after access to the last whey feeding at the end of trial 3.

Blood was sampled on the day before initiation of trial 3, and the 9th and 33rd day of that trial immediately prior to the daily whey feeding.

Veal Calves. Eighteen Holstein bull calves were fed whole milk and pelleted calf starter containing 18% crude protein until they reached an average weight of 90 kg. A 26% crude protein pelleted concentrate was then substituted for the calf starter, and liquid acid whey was slowly substituted for milk until the calves were consuming whey at a rate of 10% body weight. Details of the two pelleted concentrates are given in Tables III and IV. When whey consumption averaged 10 kg·head^{-1}·day^{-1}, the calves were grouped in threes according to body weight and calves within each group were randomly assigned to a treatment (whey containing 0.0, 0.05, or 0.10% formalin) so that all treatments were equally represented. Whey was fed individually at 0800 and 1200 h in two equal feedings supplemented by pelleted concentrate (2 kg·head^{-1}·day^{-1}) and long hay ad libitum. Unconsumed whey was weighed back at 1630 h each day, and total intake for each animal was determined. The calves were weighed weekly, the amount of whey offered being maintained at a level of 10% body weight. The day before slaughter, the calves were fed a single feeding of whey at 0800 h. All feed was withdrawn at 1200 h.

Table III. Composition of Concentrate Diets Fed When Calves Were Consuming Whole Milk and When They Were Consuming Whey

ingred/ton DM	whole milk	whey
alfalfa (dehyd, ground)		50.0
wheat		75.0
corn		310.0
oats	400.0	
barley	300.0	
soybean meal	200.0	364.0
rapeseed meal		30.0
distillers grains		50.0
molasses	40.0	50.0
bentonite		12.5
meat meal		30.0
limestone		7.5
sodium chloride		7.5
dical phosphate	30.0	7.5
mineral–vitamin mix	20.0[a]	6.0[b]

[a] Provided the following per kilogram of diet: vitamin A, 300 000 IU; vitamin D, 6000 IU; vitamin E, 600 IU; phosphorus, 170.0 g; calcium, 240.0 g; magnesium, 15.0 g; iodine, 500 mg; cobalt, 160 mg; manganese, 2.0 g. [b] Provided the following per kilogram of diet: calcium, 13.6 g; phosphorus, 4.6 g; magnesium, 2.0; cobalt, 0.279 mg; copper, 1.76 g; manganese, 50 mg; zinc, 50 mg; iodine, 1.5 mg; selenium 0.20 mg; vitamin A, 300 000 IU; vitamin D, 301 IU; vitamin E, 25 IU

Table IV. Nutrient Composition of Concentrate Diets Fed When Calves Were Consuming Whole Milk and When They Were Consuming Whey

	% total dry matter	
nutrient	whole milk	whey
crude protein	18	26
ether extract	3.5	2.4
crude fiber	4.8	4.1
calcium	0.80	0.88
phosphorus	0.69	0.71
TDN	72.2	73.3
ME, Mcal/ton	1090	1150

Two calves from each treatment group were slaughtered each week over a 3-week period. Tissue samples were therefore obtained at 81, 88, and 95 days after the initial feeding of formalin-treated whey. The slaughtering schedule facilitated the rapid sample handing necessary to analyze fresh tissue and allowed all calves to achieve a weight of approximately 225 kg. Blood samples were collected the day before slaughter, immediately prior to the whey feeding at 1200 h. Thin sections of muscle (longissimus dorsi at the last rib), kidney, liver, and heart tissues were obtained at the abattoir and immediately placed on dry ice.

Extraction Procedure. Milk sample preparation and analysis by gas chromatography were carried out as described previously (Buckley et al., 1986). Whole blood (approximately 3 mL) and slices of fresh or frozen tissue

Table V. Average Concentration of CH_2O (mg/kg) in Milk from Cows Consuming Various Levels of Formalin in Formalin-Treated Whey[a]

formalin level added to whey, % total wt	day of trial[b]									
	2	3	4	5	6	13	20	27	34	SEM
0.00	<DL[c]	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	
0.05	0.040	0.038	<DL	<DL	0.026	<DL	0.063	0.028	0.052	0.020
0.10	0.110	0.069	0.072	0.054	0.107	0.095	0.097	0.081	0.094	0.031
0.15	0.209	0.179	0.223	0.221	0.182	0.190	0.219	0.256	0.193	0.060

[a] All values are corrected to account for the reagent blank, $n = 6$. [b] Formalin-treated whey was introduced on day 1. [c] Less than detection limit (DL). DL = 0.026 mg/kg.

(wet mass 2.0–4.0 g) were weighed into Taylor tubes and extracted in the same manner as the milk samples. Frozen tissues were quickly introduced into the extraction reagent to prevent moisture loss.

Milk and blood samples were extracted within 0.5 h of collection. Representative subsamples of longissimus muscle tissue were subjected to the extraction procedure within 1 h of slaughter. Kidney, liver, heart, and the remaining muscle tissues were frozen at −4 °C until extraction could be performed.

For the analysis of blood samples only, gas chromatographic injector and column temperatures were decreased from 270 and 260 °C to 250 and 235 °C, respectively.

Statistical Analysis. In the trial with lactating cows, significant treatment effects were determined by analysis of variance utilizing a split-plot design (Little and Hills, 1972). There were six observations within each treatment, which in turn represented repeated observations within each trial. All other statistical analysis were one-way or two-way analyses of variance. Differences between treatment means were tested for significance by orthogonal comparison of means (Little and Hills, 1972)).

RESULTS

The concentrations of CH_2O determined in milk are given in Table V. Previous experimental results indicated that endogenous levels of CH_2O cannot be detected in milk by this analytical method due to a small amount of contamination in the derivatizing reagent mixture (Buckley et al., 1986). Feeding formalin-treated whey resulted in the excretion of a measurable amount of CH_2O in milk with a few exceptions. On days 3, 4, and 13 of trial 1, average milk CH_2O concentration could not be reliably determined for cows ingesting 0.05% formalin in whey because CH_2O could only be detected in the milk of three of the six cows. As the level of CH_2O intake increased, the concentration of CH_2O found in the milk increased ($P < 0.01$). There was no significant ($P > 0.05$) effect due to day of collection, indicating that milk concentrations of CH_2O did not increase over time. Average total CH_2O excreted in the morning milk during the experimental period was calculated for trials 1–3 and was found to be 0.53 ± 0.17, 1.41 ± 0.20, and 2.80 ± 0.24 mg, respectively. On the basis of average morning milk yields of 16.1, 14.9, and 12.8 kg for trials 1–3, respectively, the amount of CH_2O appearing in the milk was 3.8×10^{-3}, 5.1×10^{-3}, and 6.7×10^{-3}% of the ingested CH_2O in each trial. Formaldehyde could not be detected in milk collected prior to the initiation of each trial or 46 h after access to the last formalin-treated whey feeding.

The concentrations of CH_2O determined in the blood of the cows are illustrated in Figure 1. Analysis of variance performed on data obtained from blood CH_2O determinations is summarized in Table VI. Mean comparisons revealed that the blood CH_2O concentrations of cows consuming formalin-free whey and those consuming whey containing 0.15% formalin were not significantly ($P > 0.05$) different prior to initiation of the feeding trial or on day 9 of the trial. On day 33, the difference in average CH_2O concentrations of blood of control cows and cows consuming treated whey was significantly ($P < 0.05$) different. However, there was also a significant ($P < 0.05$) difference between control cows on day 9 of sampling compared to controls on day 33. This finding suggests that blood CH_2O concentrations in cows vary with time.

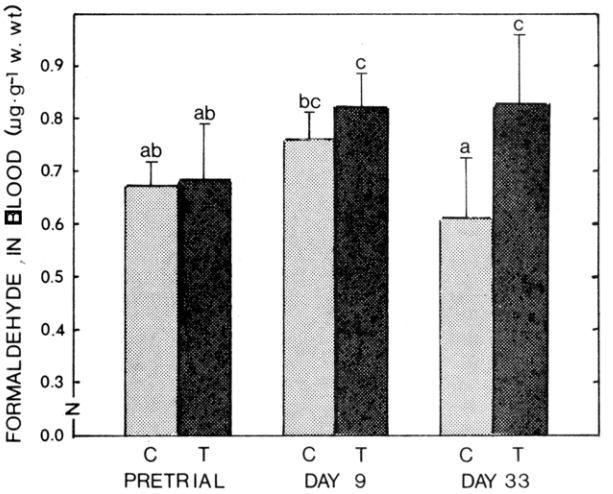

Figure 1. Concentration of formaldehyde (means ± SE, $n = 6$) in blood of cows designated as controls (C) and cows assigned to whey containing 0.15% formalin (T). CH_2O levels were measured prior to initiation of the feeding trial and on days 9 and 33 of the trial. Treatment means bearing different letters (a–c) differ significantly ($P < 0.05$).

Table VI. Analysis of Variance Table for Concentrations of CH_2O in Cattle Blood

variation source	degrees of freedom	sum of squares	mean square	F	signif level, P
total	35	0.5413			
sampling day (S)	2	0.0799	0.0400	3.8835	<0.05
treatment group (T)	1	0.0888	0.0888	8.6214	<0.01
S × T	2	0.0632	0.0316	3.0680	>0.05
residual	30	0.3094	0.0103		

Table VII shows average ($n = 6$) whey intakes and average CH_2O intakes (g) for each treatment over the entire calf-feeding trial and for the last day of the trial only. There was a significantly ($P < 0.05$) higher CH_2O concentration in fresh muscle tissue of calves consuming whey treated with 0.10% formalin compared to tissue from control calves (Table VIII). All other differences in tissue levels of CH_2O were not significant ($P > 0.05$). Variability in tissue concentrations of CH_2O due to sampling day was not significant ($P > 0.05$).

DISCUSSION

Significant ($P < 0.01$) transfer of ingested CH_2O to milk occurred at all levels of formalin fed to cows (Table V). Variability among cows in milk CH_2O concentration was expected because although all cows consumed 75 kg of

Table VII. Average Intake of Formalin-Treated Whey and Formaldehyde (CH_2O) by Dairy Calves during the Experimental Period and the Day Prior to Slaughter[a]

item	% formalin in whey		
	0.0	0.05	0.10
av whey intake,[b] kg·head^{-1}·day^{-1}	13.39 ± 2.74	13.04 ± 2.55	12.18 ± 3.10
whey intake last day of trial, kg·head^{-1}	15.34 ± 2.67	14.03 ± 2.55	13.87 ± 3.88
av CH_2O intake,[a] g·head^{-1}·day^{-1}	0.00	2.41 ± 0.047	4.50 ± 1.15
CH_2O intake day before slaughter, g·head^{-1}	0.00	2.60 ± 0.48	5.13 ± 1.43

[a] Average intakes of formalin-treated whey and formaldehyde were calculated for three slaughter groups of calves. Two calves from each treatment group (0.0, 0.05, and 0.10% formalin in whey) were slaughtered at 81, 88, and 95 days after the initial feeding of formalin-treated whey. [b] Average ± standard error.

Table VIII. Average Concentration of Formaldehyde (μg/g) in Tissues from Calves Consuming Various Levels of Formalin in Formalin-Treated Whey[a]

tissue	level of formalin, %			SEM
	0.00	0.05	0.10	
blood (fresh)	0.512	0.532	0.521	0.083
heart (frozen)	0.550	0.632	0.570	0.145
kidney (frozen)	1.373	1.701	1.541	0.313
liver (frozen)	3.623	4.082	3.478	0.621
muscle (fresh)	0.178[a]	0.206[ab]	0.256[b]	0.045
muscle (frozen)	0.133	0.155	0.188	0.033

[a] Average formaldehyde concentrations (n = 6) in tissues were determined for three slaughter groups of calves. Two calves from each treatment (0.0, 0.05, and 0.10% formalin in whey) were slaughtered at 81, 88, and 95 days after the initial feeding of formalin-treated whey. All samples of each frozen tissue type were analyzed simultaneously. Fresh muscle samples for each slaughter group were analyzed on each slaughter day. All blood samples were collected and analyzed simultaneously. Numbers in the same row that do not have a common letter in their superscripts (a,b) differ ($P < 0.05$).

whey, rate of consumption of the whey varied from a few minutes for some cows to 7 h for others. It is possible that CH_2O concentrations may have been higher in the milk from the afternoon milking, by which time the cows had consumed their daily whey allotment. Due to experimental constraints and the need for rapid analysis, it was not feasible to collect and extract milk from the afternoon milking. Preliminary experiments in this laboratory indicated that overnight refrigeration of milk samples spiked with known quantities of CH_2O resulted in low recoveries of CH_2O extracted from milk the following day. This finding is supported by the work of Tome et al. (1985) who found that reactions involving CH_2O, tyrosine, and lysine resulted in the formation of acid-resistant bonds and that of Florence and Milner (1981) who observed that CH_2O recoveries in milk decreased upon storing CH_2O-spiked samples for 7 days.

Discrepancies in the literature with regard to the appearance of CH_2O in milk of animals consuming CH_2O-treated feeds may be due in part to the form in which the CH_2O was ingested. There is some indication that CH_2O complexed with urea is not transferred to milk as readily as free CH_2O (Syrjälä-qvist and Setälä, 1982b). In feeding trials with sheep, Mills et al. (1972) observed that the proportion of ^{14}C recovered in expired air and feces appeared to be related to the reaction time between $^{14}CH_2O$ and casein prior to ingestion of the radioactive supplement. A longer reaction time resulted in a lower amount of radioactivity in expired air and a higher amount in the feces (Mills et al., 1972), indicating decreased availability of CH_2O for metabolism by rumen bacteria and lower gut digestive processes.

The concentration of CH_2O determined in blood of dairy cows was lower than that reported in rats and humans (Heck et al., 1985). Differences in blood CH_2O concentrations between monogastrics and ruminants likely reflect differences in metabolism of CH_2O. The blood of monogastrics and ruminants differs widely in the concentration of many constituents. Like the present authors, Heck and his associates (1985) found that the blood CH_2O concentration of the subject varied with time.

Ingesting CH_2O at a level of 41.6 g·day^{-1} did not appear to increase the CH_2O concentration in the blood of cows when samples were collected 23 h after last ingestion of the chemical (Figure 1). Likewise, blood CH_2O concentrations found in calves consuming approximately 2.41 and 4.50 g·day^{-1} of CH_2O did not differ ($P > 0.05$) from control values (Table VIII).

The endogenous concentrations of CH_2O found in kidney, muscle, and liver tissues (Table VIII) were much lower than those reported for sheep (Mills et al., 1972) and pigs (Florence and Milner, 1981) as determined by chromotropic acid assay. Florence and Milner (1981), who used a technique similar to that of Mills et al. (1972), noted that some charring of the sample occurred during steam distillation prior to reaction with with chromotropic acid, resulting in significant increases in CH_2O values. Therefore, it might be expected that chemical assays that are subject to such interferences are unreliable for determining trace levels of CH_2O in biological systems. Liver CH_2O concentrations in calves were very similar to those reported for rats determined by GC/MS after in situ derivatization with (pentafluorophenyl)hydrazine (Heck et al., 1982).

Lower CH_2O concentrations in frozen muscle tissue compared to fresh tissue indicate that some of the CH_2O may have been metabolized or irreversibly bound to muscle protein, thereby reducing recovery of analyzable CH_2O in frozen tissue. Volatilization as an avenue of tissue CH_2O loss seems improbable since CH_2O readily polymerizes at refrigerated temperatures, thereby becoming less volatile.

Although there is some indication in rats and humans that toxicity due to CH_2O inhalation is unlikely to occur at sites distant from the nasal mucosa (Heck et al., 1985), our research indicates that ingestion of CH_2O by cattle results in elevated levels of CH_2O in milk and muscle tissue. However, the amounts of CH_2O found in the present study are much less than those found in certain human food and beverage products such as beer and soft drinks (0.1–1.5 and 7.4–8.7 mg/kg, respectively; Lawrence and Iyengar, 1983), ripened Provolone cheese and the outer layer of smoked ham (26 and 224–267 mg/kg, respectively; Brunn and Klostermeyer, 1984), and coffee (3.4–16.3 ppm; Hayashi et al., 1986). It is likely that storage and pasteurization of milk from cows ingesting formalin would result in a reduction of free CH_2O through irreversible binding with milk protein.

ACKNOWLEDGMENT

This research was funded by Agriculture Canada under the auspices of the Feed from Waste Program. We express our gratitude to Mike Yusko and the Fraser Valley Milk Producers Co-operative Association for supplying fresh whey in support of the study.

Registry No. CH_2O, 50-00-0.

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Received for review July 7, 1987. Revised manuscript received March 25, 1988. Accepted May 7, 1988.

Isolation and Characterization of Low Molecular Weight Protein from Mustard (*Brassica juncea*)

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The low molecular weight protein fraction from *Brassica juncea*, the mustard seed, has been purified to homogeneity by ammonium sulfate fractionation followed by gel filtration on a Sephadex G-75 column. The protein contains 0.2% carbohydrate, is free from phosphorus, and is rich in its available lysine content (6.7 ± 0.4%). The protein has a sedimentation coefficient of 1.7 S and molecular weight of 22 900 ± 2000 and consists of two polypeptide chains of molecular weights 13 000 and 12 000. The protein has a low content of aromatic amino acids and is free from chromogenic impurities like phenolic acids and nucleic acids; it has an absorption maximum at 278 nm and a shoulder at 288 nm. The fluorescence emission maximum is at 335 nm. The optical rotatory dispersion spectrum indicates a trough at 232 nm with −6000° cm^2 dmol^{-1} of molecular rotation. The near-ultraviolet and far-ultraviolet circular dichroism measurements indicate that the protein differs from the high molecular weight protein fraction in terms of its tertiary and secondary structure.

Mustard (*Brassica juncea*) is one of the major oilseeds of India. The defatted meal is of nutritionally superior quality and can be incorporated in human and animal food and feed after the removal of glucosinolates (Ohlson, 1985). An understanding of the chemical and physicochemical properties of the proteins and their interactions with toxic and chromogenic components may lead to better methods of processing and detoxification. Mustard proteins consist of two protein fractions: a high molecular weight protein fraction (12S fraction) constituting about 25% and a low molecular weight protein fraction constituting about 70% of the total proteins (Gururaj Rao et al., 1978). The low molecular weight protein fraction consists of three to four proteins that are very similar in size but differ in their electrophoretic mobility.

There are reports in the literature regarding the isolation and characterization of low molecular weight protein from *Brassica napus* L., the rapeseed (Bhatty et al., 1968; Swanljung, 1972; Lonnerdal and Janson, 1972; Mackenzie and Blakely, 1972; Schwenke et al., 1973; Simard and Boulet, 1978; Raab and Schwenke, 1984). Crouch et al. (1983) have reported the amino acid sequence of this protein and the DNA sequence of the gene. The various methods that have been reported for the isolation of 2S protein from rapeseed involve the removal of 12S protein by ammonium sulfate precipitation or gel filtration on Sephadex gels and fractionation of the low molecular weight protein by repeated exclusion chromatography on Sephadex gels followed by resolving the proteins that are differing in charge though similar in size with ion-exchange chromatography. Raab and Schwenke (1984) have isolated a low molecular weight protein fraction by means of fractional precipitation and dissolution with ammonium

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0021-8561/88/1436-1150$01.50/0