Electrophoretograms of Peas and Skim Milk Proteins Hydrolyzed in Vitro with Pepsin and Rennin

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Three protein products, pea protein isolate, pea protein concentrate, and reconstituted skim milk, were hydrolyzed in vitro with commercial pepsin and rennin for 0.25–22 h. The peptide bond hydrolysis was determined by reaction with 2,4,6-trinitrobenzenesulfonic acid. The order of hydrolysis of the three proteins by pepsin was PPI > PPC > SM and by rennin was SM > PPI > PPC. In general, the pea protein isolate and pea protein concentrate proteins appeared to be more susceptible to pepsin, and skim milk proteins, as expected, appeared to be more susceptible to rennin. The sodium dodecyl sulfate–polyacrylamide gel electrophoretic patterns of PPI and PPC showed that proteins having a molecular weight between 70 000 and 300 000 were preferentially hydrolyzed, although several protein bands were still present after 22 h of hydrolysis, suggesting the presence of resistant proteins or peptides. The preferentially hydrolyzed proteins were probably acidic proteins or subunits, and their relative ease of hydrolysis may be related to their chemical composition and/or structure.

Dry (field) peas are well established as one of the protein crops in Western Canada. Commercial products of peas such as pea protein concentrate (PPC) and pea protein isolate (PPI) are expected to be increasingly used in animal feeds and ultimately in human foods to supplement dietary protein. Thus, PPC has been used as a partial milk replacer for preruminant calves, but its protein digestibility has been low (Bell et al., 1974). Although protein digestibility of PPI was greatly superior to that of PPC, it was not equal to the control diet, reconstituted skim milk (SM) (Bhatty and Christison, 1980). Higher contents of starch and oligosaccharides in PPC were probably largely responsible for its lower protein digestibility; nevertheless, other factors must be reponsible for the differences in the protein digestibility of PPC and PPI and of PPI and SM.

Whereas protein quality of a feed or food may be a function of its amino acid composition and proportion, protein digestibility is a rate measurement of protein hydrolysis by proteolytic enzymes (Kakade, 1974). It is well documented that legume proteins have lower in vivo digestibilities than animal proteins (Tobin and Carpenter, 1978; Rockland and Radke, 1981). Although heating may considerably improve the digestibility of some legume protein, others resist thermal denaturation and improvement in digestibility. Seidle et al. (1969) reported bean globulin protein to be highly resistant to in vitro hydrolysis by a number of proteolytic enzymes even after denaturation with heat or urea. A more recent study (Liener and Thompson, 1980) reported native GI protein (vicilin) of *Phaseolus vulgaris* to be highly resistant to digestion both in vivo and in vitro unless subjected to thermal treatment. Thus, not only is there a need to improve the digestibility of legume protein but also there is a need to understand the biochemical phenomena involved in the reduced digestibility of legume protein compared to that of animal protein.

The present study reports on the electrophoretograms of PPI, PPC, and SM proteins hydrolyzed in vitro with pepsin and rennin. The objective was to determine the rates of hydrolysis of the three proteins and to identify protein components which were hydrolyzed preferentially or were relatively resistant to hydrolysis. It was thought that some of these components may be responsible, at least in part, for the observed differences in the in vitro digestibility of the three proteins.

EXPERIMENTAL SECTION

Pea protein concentrate (HI PRO 55) prepared by pin milling and air classification was obtained from PRO-STAR Mills, Saskatoon, Saskatchewan. It contained 61.2% protein and 5.5% starch on a dry basis. The comparative composition of PPC, PPI, and SM has been described elsewhere (Bhatty and Christison, 1980). PPI was prepared from the PPC by a procedure described previously (Bhatty, 1982a). The PPC and PPI contained on a dry basis 9.1 and 14.2% nitrogen, respectively. Rennin (chymosin, EC 3.4.23.4) from calf stomach and pepsin (EC 3.4.4.1) from hog stomach were purchased from the Sigma Chemical Co., St. Louis, MO. 2,4,6-Trinitrobenzenesulfonic acid (TNBS) was purchased from ICN Nutritional Biochemicals, Montreal, Quebec, and was recrystallized as described by Fields (1971).

In Vitro Hydrolysis of PPI, PPC, and SM. The substrate concentrates were PPI 2.2%, PPC 3.5%, and SM 5.8%, all containing equal amount of nitrogen, and were dispersed in 60 mM HCl for hydrolysis with pepsin (pH 2.1) and in 30 mM HCl for hydrolysis with rennin (pH 3.4). The enzyme concentrations were pepsin 0.02 mg/mL in 10 mM HCl and rennin 0.25 mg/mL in 0.1 mM HCl prepared freshly at the time of assay. The digestion mixture contained 5.0 mL of the substrate and 1.0 mL of the enzyme solution and was incubated at 37 °C. At appropriate time intervals, a 2.0-mL aliquot of the digestion mixture was withdrawn, mixed with 4.0 mL of hot 0.2125 M sodium phosphate buffer, pH 8.2, containing 3.0% (w/v) sodium dodecyl sulfate ($NaDodSO_4$), stoppered, and kept at 80 °C for 30 min to inactivate the enzyme. Appropriate controls containing only enzyme or substrate were prepared separately under identical conditions. The hydrolysates were stored at 4 °C until analyzed for peptide bond hydrolysis by the method of Adler-Nissen (1979) as modified by Patel and Bhatty (1982) or electrophoresed by the method of Weber et al. (1968) at a gel concentration of 7.5% and an acrylamide to methylenebis(acrylamide) ratio of 37:1. The molecular weight of the protein bands was calculated from a standard curve prepared with Pharmacia standards (M_r

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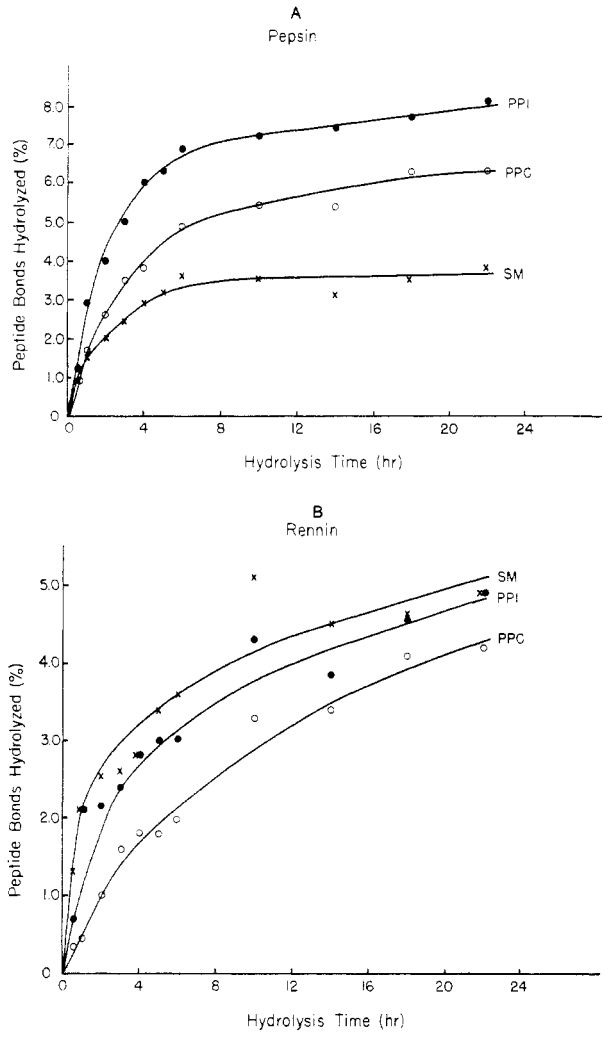

Figure 1. The peptide bond hydrolysis of pea protein isolate (PPI), pea protein concentrate (PPC), and reconstituted skim milk (SM) by pepsin (A) and rennin (B) for 0.25–22 h.

range 14 400–330 000) electrophoresed under identical conditions. The relative mobility (M) was expressed as the ratio of the distance (mm) traveled by the protein bands to that traveled by the tracking dye (100 mm).

RESULTS AND DISCUSSION

The SM, PPC, and PPI were hydrolyzed with pepsin and rennin for 0.25–22 h and the peptide bond hydrolysis measured by reaction with TNBS. The TNBS procedure was preferred over the assay procedure described previously (Bhatty, 1982a), as it was not likely to be influenced by differences in aromatic amino acid content of the three proteins or by the presence of peptides soluble in trichloroacetic acid (TCA). The TCA used in many in vitro protein digestibility studies to terminate enzyme digestion is also not satisfactory where the products of hydrolysis need to be identified as was done in this study.

Figure 1 shows that the hydrolysis of PPI, PPC, and SM was curvilinear and that the order of hydrolysis of the three proteins by pepsin was PPI > PPC > SM and by rennin was SM > PPI > PPC. It is difficult to explain the low digestibility of SM by pepsin. Skim milk contained more aromatic amino acid residues than PPI and PPC (Bhatty and Christison, 1980). These residues, if present on both sides of the peptide bond, are preferentially hydrolyzed by pepsin (Bovey and Yanari, 1960). Low digestibility of SM may partly be due to substrate inhibition of pepsin.

Figure 1 also shows that approximately 3–5% of the peptide bonds of the three substrates were hydrolyzed by rennin and 3–8% by pepsin for a hydrolysis time of 22 h. These values generally confirm the slow in vitro hydrolysis of vegetable proteins by commercial proteolytic enzymes. Romero and Ryan (1978) reported only 2–3% peptide bond hydrolysis of a bean GI protein hydrolyzed in vitro with pepsin, or chymotrypsin for 22 h. However, a faster hydrolysis of isolated soybean proteins was reported by Boonvisut and Whitaker (1976) and Lynch et al. (1977). The reason(s) for the low in vitro hydrolysis of the proteins in this study may partly be due to less than ideal enzyme to substrate ratios, since these were arbitrarily used and, in addition, in the case of pea proteins probably to their molecular size, the presence of inhibitors, and (or) their structure. The influence of these factors on in vitro protein digestibility can at best be speculated about and needs investigation.

Figure 2 shows the $NaDodSO_4$–polyacrylamide gel electrophoretic patterns of pepsin, rennin, unhydrolyzed PPI, PPC, and SM, and PPI, PPC, and SM hydrolyzed by pepsin and rennin for 0.25–22 h. Commercial pepsin (Aa, Ba, Ca) showed two protein bands, the major or the heavily stained band had an average M of 0.65 and a calculated M_r of ca. 34 000. This value compares favorably with a M_r of 34 000 for pepsin determined by sedimentation equilibrium (Bovey and Yanari, 1960). Unlike pepsin, commercial rennin showed many protein bands (Da, Ea, Fa); the major protein band corresponding to an M of 0.62 (Da, Fa) had a M_r of 36 000.

Unhydrolyzed PPI (Ab, Db) and PPC (Bb, Eb) were highly heterogeneous and contained protein components ranging in M_r in the case of PPI from 15 000 to 300 000 (M = 0.02 to 1.02) and in the case of PPC from about 11 000 to 300 000 (M = 0.02 to 1.18). The difference in the range of M_r between the two protein products was due to removal of low molecular weight protein components from PPC during the preparation of PPI. However, a large range in the molecular weight of PPC and PPI proteins was expected as PPC and PPI contain both albumin and globulin proteins (Bhatty, 1982a). Pea albumin proteins dissociate in $NaDodSO_4$–polyacrylamide gel electrophoresis into at least 24 protein components (Bhatty, 1982b). In addition, globulin protein of peas contains many acidic and basic subunits (Krishna et al., 1979). In contrast, SM (Cb, Fb) contained relatively fewer protein components; the five heavily stained protein bands ranged in M_r from 13 500 to 65 000 (M = 0.34 to 1.08) corresponding to major milk proteins (Brunner, 1977).

The electrophoretic patterns in Figure 2 also indicate that unhydrolyzed PPI (Ab, Db) did not contain any protein bands having an M greater than 1.04 or a M_r lower than 15 000. In contrast, PPI hydrolyzed with pepsin for 0.25–22 h (A) contained at least three heavily stained protein bands which had an M greater than 1.04 or a M_r lower than 15 000. These M_rs can only be approximate as they were obtained from the extrapolated portion of the standard curve. Thus, proteins or peptides having a M_r between 1000 and 15 000 were cleaved from the PPI after only 15 min of hydrolysis. It is also possible that some of these peptides were formed from smaller peptides by aggregation. Although there was no further increase in the number of protein bands having a M_r of 15 000 or lower after 0.25 h of hydrolysis, this may not suggest that further cleavage of PPI by pepsin did not take place. Figure 1 shows that the peptide bond hydrolysis of PPI by pepsin increased about 7-fold between hydrolysis times of 0.25 and 22 h. However, because of the complexity of the electrophoretic patterns, it was difficult to identify the origin of the products of hydrolysis. Therefore, it seemed more

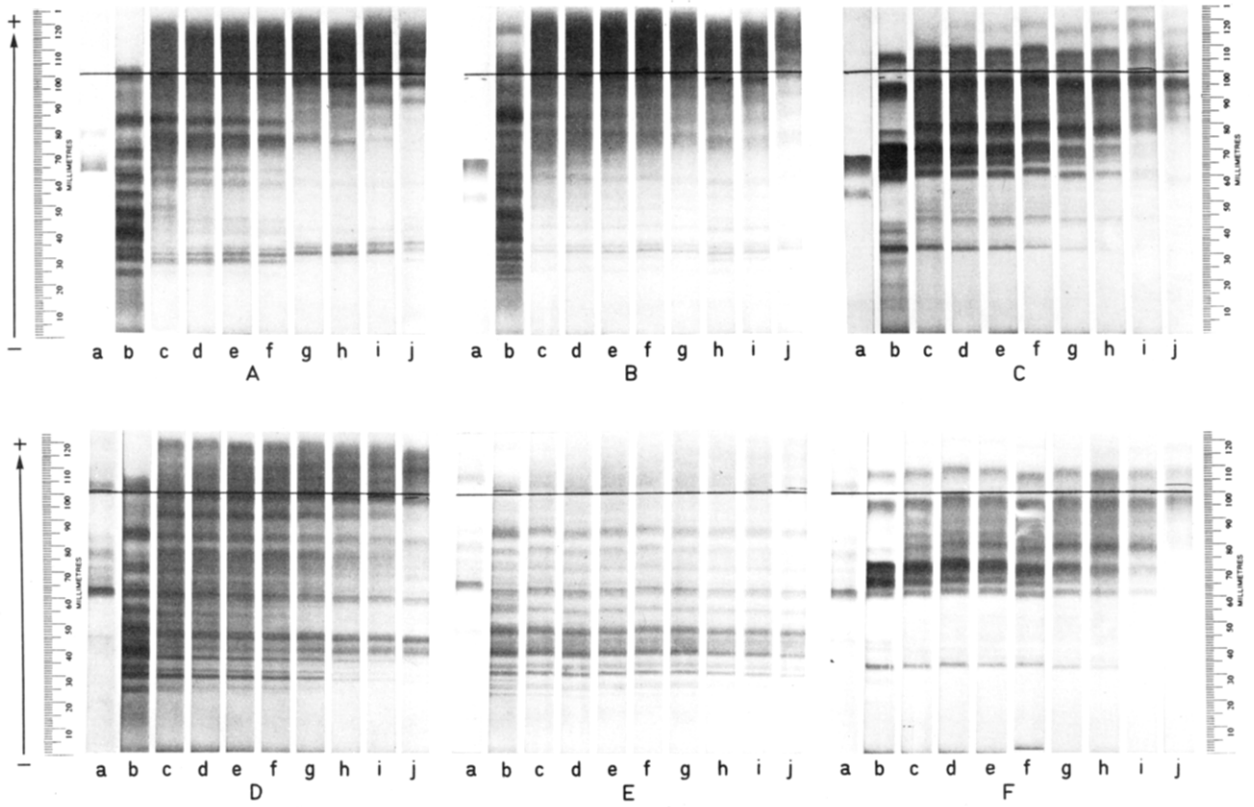

Figure 2. Electrophoretograms of PPI (A), PPC (B), and SM (C) hydrolyzed by pepsin and of PPI (D), PPC (E), and SM (F) hydrolyzed by rennin for 0.25–22 h. Gels: Aa, Ba, and Ca, pepsin; Da, Ea, and Fa, rennin; Ab and Db, unhydrolyzed PPI; Bb and Eb, unhydrolyzed PPC; Cb and Fb, unhydrolyzed SM; c, d, e, f, g, h, i, and j, hydrolyzed by pepsin (A, B, C) and rennin (D, E, F) for 0.25, 0.50, 0.75, 1.0, 2.0, 4.0, 6.0, and 22 h, respectively.

meaningful to interpret the electrophoretograms by identifying the disappearance of protein bands from the unhydrolyzed proteins.

Although unhydrolyzed PPI (Ab, Db) and PPC (Bb, Eb) contained large M_r protein bands at an M of 0.02 (near the origin) and 0.14, there did not appear to be any evidence of these proteins being present in any of the subsequent gels after hydrolysis with pepsin or rennin. Since these were relatively the largest M_r proteins present in PPI and PPC (M_r 100 000–300 000), their disappearance at the earliest hydrolysis time suggested that these were hydrolyzed preferentially. This may suggest that larger M_r proteins had more peptide bonds susceptible to hydrolysis compared to some of the smaller M_r proteins which may be more tightly folded and may have higher enzyme inhibitory tendencies. The gel patterns of PPI hydrolyzed by pepsin (A) further showed that a protein band with an M of 0.23 (M_r 80 000) was faintly visible up to a hydrolysis time of 1 h (Af), after which there was no trace of this protein band in any of the subsequent gels. Similarly, the gel pattern of PPI hydrolyzed by rennin (D) showed that a protein band having similar M and M_r was present up to a hydrolysis time of 2 h (Dg), after which there was no evidence of its presence. Thus, a protein band having a similar M and M_r was hydrolyzed relatively slowly by rennin as compared to that by pepsin, even though both the enzymes have similar specificities (Bovey and Yanari, 1960). An overall comparison of the hydrolysis of PPI by pepsin (A) and rennin (D) indicated that pepsin was more effective than rennin in hydrolyzing PPI proteins as fewer protein bands were present in gels Ac–j than in corresponding gels of Dc–j. A similar general pattern was apparent in the hydrolysis of PPC by pepsin and rennin (Bc–j and Ec–j). Unhydrolyzed PPI contained three other protein bands with an M of 0.46, 0.63, and 0.84 (M_r 50 000, 35 000, 23 000, respectively), which disappeared after 2 h of hydrolysis with pepsin. However, PPI contained at an M of about 0.30 (M_r 70 000) three protein bands which were most resistant to hydrolysis by pepsin as these bands were still present after 22 h of hydrolysis. Unlike PPI hydrolyzed by pepsin, PPI hydrolyzed by rennin contained more protein bands which were still intact after 22 h of hydrolysis.

The $NaDodSO_4$–polyacrylamide gel electrophoretic patterns of PPC hydrolyzed by pepsin (B) or rennin (E) were generally similar to those of PPI hydrolyzed by the same enzymes. Unhydrolyzed PPC contained at least three protein bands having an M greater than 1.0 (Bh, Eb). PPC hydrolyzed with pepsin for up to 22 h also contained three protein bands having nearly similar M, even though a number of protein bands had disappeared from the PPC during the course of the hydrolysis. Nevertheless, PPC, like PPI, also contained proteins (M = 0.30; M_r 70 000) which were resistant to pepsin, as they persisted to a hydrolysis time of 22 h. However, PPC proteins having a M_r larger than 70 000 (M = 0.30) were hydrolyzed, as they were not present in any of the gels (Bc–j). PPI contained even more proteins which were resistant to rennin; the only evidence of protein being hydrolyzed were those having an M of less than 0.20 or a M_r of around 85 000.

Unhydrolyzed SM contained only one protein band having a M greater than 1.0 (Cb, Fb) while SM hydrolyzed by pepsin contained three protein bands (Cc–j) compared to one and in some cases two protein bands in SM hydrolyzed by rennin (Fc–j). Although SM also contained proteins which were resistant particularly to pepsin or were hydrolyzed slowly by this enzyme (also shown by Figure 1), it seemed that most of the SM proteins were more susceptible to rennin than to pepsin hydrolysis. This was probably due to SM protein being the natural substrate

for rennin. However, it appeared from the gel patterns that SM proteins having a M_r greater than 59 000 (M = 0.38) were preferentially hydrolyzed, as these bands were not present in any of the subsequent gels (Cc–j and Fc–j).

The major pea proteins, legumin and vicilin, have a M_r ranging from 140 000 to 300 000; legumin dissociates into 10 or 12 subunits ranging in M_r from 20 000 to 40 000 (Derbyshire et al., 1976). However proteins or protein subunits having a M_r in the vicinity of 75–80 000, which were present both in PPI and PPC and were preferentially hydrolyzed, have not been reported in peas. Therefore, it is likely that these proteins were formed during the processing of PPI and PPC either through a random dissociation from the larger pea proteins or by aggregation of the two or more subunits through disulfide interchange or by hydrogen bonding. Although PPI and PPC proteins seemed more susceptible to pepsin than rennin (unlike SM proteins which were more susceptible to rennin than pepsin), there were many proteins present in both the substrates which were not hydrolyzed by the enzymes even after 22 h of hydrolysis. These may be termed as resistant proteins or peptides. Their lack of hydrolysis may be due to their association with nonprotein components such as sugars or to lack of susceptible bonds and structural peculiarities or to a combination of all of these and other factors. Studies are being directed on some of these aspects of in vitro hydrolysis of pea and other legume proteins.

CONCLUSION

The electrophoretograms of PPI and PPC hydrolyzed with pepsin and rennin suggested that protein components having a M_r between 70 000 and 300 000 appeared to be preferentially hydrolyzed both by pepsin and by rennin, though a number of other protein components were also not visible especially in the case of pepsin after 1.0 h of hydrolysis. In the case of SM, there was no trace of the protein bands having a M_r greater than 59 000 after a hydrolysis time of 0.25 h by pepsin or rennin. The proteins which were preferentially hydrolyzed by pepsin and rennin (M_r greater than 70 000 in PPI and PPC) may be acidic proteins or subunits. A preferential hydrolysis of acidic soybean glycinin protein by pepsin and trypsin was reported by Lynch et al. (1977) using $NaDodSO_4$–polyacrylamide gel electrophoresis. Nevertheless, the present data need corroboration after isolation of the acidic protein subunits by ion-exchange chromatography. The reason(s) for the preferential hydrolysis of the acidic protein is (are) not known but may be due to their lower hydrophobicity and to a less compact structure than that of basic proteins.

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Registry No. Pepsin, 9001-75-6; rennin, 9001-98-3.

LITERATURE CITED

Adler-Nissen, J. *J. Agric. Food Chem.* **1979**, *27*, 1256.
Bell, J. M.; Royan, G. F.; Youngs, C. G. *Can. J. Anim. Sci.* **1974**, *54*, 355.
Bhatty, R. S. *Can. Inst. Food Sci. Technol. J.* **1982a**, *15*, 101.
Bhatty, R. S. *J. Agric. Food Chem.* **1982b**, *30*, 620.
Bhatty, R. S.; Christison, G. I. *Can. I. Anim. Sci.* **1980**, *60*, 925.
Boonvisut, S.; Whitaker, J. R. *J. Agric. Food Chem.* **1976**, *24*, 1130.
Bovey, F. A.; Yanari, S. S. *Enzymes, 2nd Ed.* **1960**, *3*, 63.
Brunner, J. R. In "Food Proteins"; Whitaker, J. R.; Tannenbaum, S. R., Eds.; Avi Publishing Co., Inc.: Westport, CT, 1977.
Derbyshire, E.; Wright, D. J.; Boulter, D. *Phytochemistry* **1976**, *15*, 3.
Fields, R. *Biochem. J.* **1971**, *124*, 581.
Kakade, M. L. *J. Agric. Food Chem.* **1974**, *22*, 550.
Krishna, T.; Croy, R. R. D.; Boulter, D. *Phytochemistry* **1979**, *18*, 1879.
Liener, I. E.; Thompson, R. M. *Qual. Plant.—Plant Foods Hum. Nutr.* **1980**, *30*, 13.
Lynch, C. J.; Rha, C. K.; Catsimpoolas, N. *Cereal Chem.* **1977**, *54*, 1282.
Patel, P. R.; Bhatty, R. S., Department of Crop Science, University of Saskatchewan, Saskatoon, Canada, unpublished data, 1982.
Rockland, L. B.; Radke, T. M. *Food Technol. (Chicago)* **1981**, *35*, 79.
Romero, J.; Ryan, D. S. *J. Agric. Food Chem.* **1978**, *26*, 784.
Seidle, D.; Jaffe, M.; Jaffe, W. G. *J. Agric. Food Chem.* **1969**, *17*, 1318.
Tobin, G.; Carpenter, K. J. *Nutr. Abstr. Rev., Ser. A: Hum. Exps.* **1978**, *48*, 919.
Weber, K.; Pringle, J. R.; Osborn, M. *Methods Enzymol.* **1968**, *26*, 3.

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