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Graft Copolymerization onto Protein by the Ceric Ion Method. Studies on Grafting Site

YOHJI IMAI and YOSHIO IWAKURA,
Department of Synthetic Chemistry, Faculty of Engineering, University of Tokyo, Tokyo, Japan

Synopsis

Graft copolymerization of methyl methacrylate and acrylamide onto ovalbumin was carried out with redox system with ceric ion. Initiation of grafting occurred very rapidly, and the number of grafting sites reached a maximum after about 2 min. regardless of initial ceric ion concentration. The grafted polymer chains were separated by selective hydrolysis of the protein backbone with hydrochloric acid or protease in order to characterize the graft copolymers. The grafted polyacrylamide separated by the proteolytic digestion contained a carbohydrate residue at the end of the polymer molecule. The problem of grafting sites on ovalbumin is discussed.

Ceric ion forms very effective redox systems in the presence of organic reducing agents such as alcohols, glycols, aldehydes, acetals, thiols, esters, and carboxylic acids.[1] The use of ceric ion redox systems in the preparation of graft copolymers has not been extended beyond polyhydroxyl compounds[1,2] such as cellulose, starch, and poly(vinyl alcohol), and there is little information relating to the grafting sites. Previous works in this series dealing with cellulose[3] and poly(vinyl alcohol)[4] have shown that the grafting is likely to occur at hemiacetal and 1,2-glycol units in the case of cellulose and at the 1,2-glycol unit in the case of poly(vinyl alcohol). It appeared of interest to study grafting onto proteins by the ceric ion method and to determine the grafting sites on proteins, since proteins contain various functional groups such as hydroxyl, sulfhydryl, and amino groups which are capable of forming redox systems with ceric ion. In the work reported here the protein used was ovalbumin because pure material can be obtained easily from hen's eggs.

EXPERIMENTAL

Materials

Ovalbumin was isolated from hen's eggs in the manner described by Sørensen and Høyrup,[5] recrystallized three times from aqueous ammonium sulfate, dialyzed, and lyophilized. Reagent grade ceric ammonium nitrate from G. F. Smith Chemical Co. was used without further purification. Commercial methyl methacrylate was purified by the usual method and

distilled. The middle cut, boiling at 46–46.5°C./100 mm. Hg was retained and stored in the dark at −20°C. until used. Commercial acrylamide was recrystallized twice from ethyl acetate, m.p. 84°C. A commercial protease (Pronase-P) isolated from a culture medium of *Streptomyces griseus* was purchased from Kaken Kagaku Co.

Method of Grafting

Methyl Methacrylate. An aqueous solution of ovalbumin and methyl methacrylate were placed together in a three-necked flask equipped with a condenser and a gas inlet tube. After nitrogen was bubbled through the solution for 40 min. at 30°C., grafting was started by adding the ceric ammonium nitrate solution in 1*N* nitric acid. Polymerization was terminated by adding methanol, and the mixture was dialyzed against running tap water, and then was evaporated to dryness *in vacuo*. The polymerization product obtained was washed thoroughly with acetone, dried *in vacuo*, and weighed.

Acrylamide. Grafting was carried out in the manner similar to the preceding section except that the polymerization product was dialyzed against running tap water, evaporated to dryness *in vacuo* and weighed.

Hydrolysis of the Protein Backbone with Hydrochloric Acid

Samples of the ovalbumin–methyl methacrylate graft copolymers (0.1–0.3 g.) were treated with 2 ml. of glacial acetic acid and 4 ml. of 6*N* hydrochloric acid at 100–110°C. for 9 hr. The precipitates were filtered, washed with water and methanol, and then were dissolved in acetone, reprecipitated with methanol, dried *in vacuo*, and weighed. The weight loss by this treatment was virtually identical with the weight of protein backbone. Infrared spectra of the products were identical with that of poly(methyl methacrylate).

Digestion of the Protein Backbone with Trypsin

Samples of the ovalbumin–acrylamide graft copolymers (0.1–0.3 g.) were digested with 10 mg. of trypsin in 50 ml. of 0.1*M* phosphate buffer of pH 7.6 at 37°C. for 3 days. The gellike graft copolymers became soluble as the digestion proceeded. After digestion the resulting solution was dialyzed against running tap water for 3 days. Polyacrylamide were precipitated with methanol.

Ovalbumin–methyl methacrylate graft copolymers were not affected by trypsin.

Digestion of the Protein Backbone with Pronase-P

Samples of the ovalbumin–acrylamide graft copolymers (0.1–0.3 g.) were digested with 10 mg. of Pronase-P in 50 ml. of 0.1*M* borate buffer of pH 8.0 containing 0.01*M* calcium acetate as an activator for the pro-

tease. Polyacrylamide was isolated similarly as in the preceding experiment. The infrared spectra of the isolated polymers were identical with that of polyacrylamide.

The protein backbone of ovalbumin–methyl methacrylate graft copolymers was digested by Pronase-P under similar conditions.

Molecular Weight Determination of the Separated Polymers

The intrinsic viscosity of the poly(methyl methacrylate) samples separated by hydrochloric acid treatment was determined in benzene at 30°C. The number-average molecular weight was calculated from the equation:[6]

$$[\eta] = 8.69 \times 10^{-5} \bar{M}_n^{0.76}$$

The weight-average molecular weights of the polyacrylamide separated by the enzymatic digestion were calculated from intrinsic viscosities measured in 1*N* aqueous sodium chloride at 30°C. by using the equation:[7]

$$[\eta] = 3.73 \times 10^{-4} \bar{M}_w^{0.66}$$

Pretreatment of Ovalbumin with Ceric Ammonium Nitrate

A solution of 1 g. of ovalbumin in 30 ml. of distilled water was heated with swirling at 95–100°C. for 3 min. To this heat-denatured solution, 170 ml. of distilled water was added. After nitrogen was bubbled through the solution for 15 min., 0.11 g. of ceric ammonium nitrate in 4 ml. of 1*N* nitric acid was added, and the mixture was allowed to react at 30°C. for 15 min. under nitrogen. The precipitate was filtered, washed with water and methanol, and dried *in vacuo*. The dried materials were ground in an agate mortar and sieved to study grafting of uniform particles.

Pretreatment of Ovalbumin with Paraperiodic Acid

A solution of 1 g. of ovalbumin in 25 ml. of distilled water was heated with swirling at 95–100°C. for 3 min., cooled to 0°C., followed by addition of 0.1 g. of paraperiodic acid in 5 ml. of water, and then allowed to stand in dark for 24 hr. at 0–5°C. The precipitate was subjected to the same treatment as described above.

Pretreatment of Ovalbumin with Iodoacetic Acid

A solution of 1 g. of ovalbumin in 25 ml. of distilled water was heated at 95–100°C. for 3 min. To this was added 5 ml. of sodium iodoacetate solution which was prepared by neutralizing 0.1 g. of iodoacetic acid (recrystallized from *n*-hexane) with 0.1*N* sodium hydroxide. The mixture was left for 20 hr. at room temperature. Subsequent treatment was as described above.

Preparation and Separation of the Grafted Polyacrylamide from Ovalbumin Substrate for Carbohydrate Detection

Ovalbumin–acrylamide graft copolymer was digested with Pronase-P as described above. After digestion the solution was concentrated under vacuum below 40°C. down to a volume about one-tenth of the original solution. The digestion products were precipitated with methanol. The digestion was repeated once and the precipitate was subjected to deproteinization by 5% trichloroacetic acid. After the removal of trichloroacetic acid by ether extraction, the aqueous solution was dialyzed against distilled water for 30 days with cellulose tubing (Visking Co.) to remove low molecular weight materials. Polyacrylamide was precipitated with methanol, filtered on a glass filter, and dried *in vacuo*.

Determination of Carbohydrates Attached to the Polyacrylamide

The polyacrylamide obtained as described in the preceding section was heated 1*N* hydrochloric acid at 90–100°C. for 7 hr. The solution was evaporated to dryness with a rotary evaporator and the residue was transferred with some distilled water to cellulose tubing and dialyzed against distilled water. The outside water was changed several times and combined solutions were evaporated to dryness under reduced pressure. The residue was tested for carbohydrates; the anthrone reaction[8] for neutral sugars and the Elson-Morgan reaction[9] for amino sugar were used.

RESULTS

The preliminary experiments using various kinds of proteins showed that rapid grafting occurred onto ovalbumin, bovine serum albumin, and gelatin, but that grafting was slow onto silk, trypsin, bacterial amylase, and Pronase-P under the same conditions.

Table I shows typical results of grafting of methyl methacrylate onto native, heat-denatured, ceric ion-treated, periodic acid-treated, and iodoacetic acid-treated ovalbumin. The percentage weight increase was calculated as [(total weight of graft copolymer-weight of ovalbumin)/weight of ovalbumin] × 100. The molecular weight of grafted poly(methyl methacrylate) (PMMA) obtained by the hydrolysis of ovalbumin backbone with hydrochloric acid is given in Table I. The number of grafting sites per mole of ovalbumin was calculated as [(weight of grafted PMMA/molecular weight of grafted PMMA)/(weight of used ovalbumin/molecular weight of ovalbumin)] and is also given in Table I. The molecular weight of the ovalbumin is 45,000.[10]

In native ovalbumin very rapid grafting occurred, and the solution became gradually turbid and the polymer precipitated. Weight increase per cent and molecular weight of grafted PMMA increased markedly with polymerization time, while the apparent number of grafting sites increased to a maximum, and then decreased and nearly leveled off. This is clearly shown in Figure 1.

TABLE I
Grafting of Methyl Methacrylate onto Ovalbumin[a]

Sample	Time, min.	Weight increase, %	Mol. wt. of grafted PMMA × 10^{-5}	No. of grafting sites, mole/mole
Native ovalbumin	0.2	121	1.0	0.55
	2	388	1.8	0.98
	5	509	2.4	0.96
	10	827	5.4	0.69
	15	988	6.2	0.73
	20	1397	8.7	0.73
	45	1590	9.6	0.75
Heat-denatured ovalbumin in aqueous solution	5	199	1.4	0.64
	10	248	2.1	0.54
	15	326	3.2	0.46
Heat-denatured and dried ovalbumin (100–220 mesh)	5	71	1.5	0.21
	10	95	2.1	0.20
	15	86	2.0	0.20
Ceric ion-treated ovalbumin (100–220 mesh)	5	142	3.8	0.17
	10	221	7.8	0.13
Periodic acid-treated ovalbumin (100–220 mesh)	5	101	3.5	0.13
	10	185	4.9	0.17
	15	249	5.4	0.21
Iodoacetic acid-treated ovalbumin (100–220 mesh)	5	112	2.1	0.24
	10	168	2.8	0.27
	15	163	2.6	0.28

[a] At 30°C.; ovalbumin = 0.100 g.; [ceric ammonium nitrate]$_0$ = 0.67 × 10^{-3} mole/l.; [nitric acid] = 0.01 mole/l.

In heat-denatured and dried ovalbumin no marked change in percentage weight increase, molecular weight of grafted PMMA, and number of grafting sites was observed within 15 min. Ovalbumin which was heat-denatured in aqueous solution before grafting showed behavior intermediate between those of native ovalbumin and heat-denatured and dried ovalbumin.

With periodic acid-treated ovalbumin an increase in per cent weight increase and molecular weight of grafted PMMA was observed, accompanied by a slight decrease in the number of grafting sites compared with the parent ovalbumin.

The results of grafting of acrylamide onto ovalbumin are shown in Table II. The reaction mixture turned gradually into gel as the grafting proceeded.

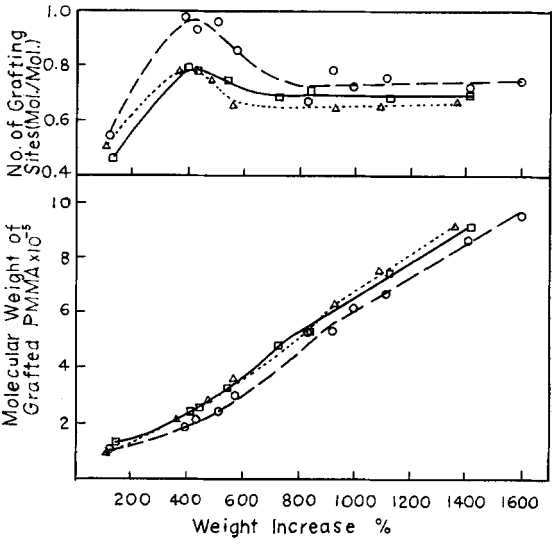

Fig. 1. Plots of molecular weight of grafted PMMA and number of grafting sites vs. per cent weight increase for grafting of methyl methacrylate onto ovalbumin for various initial ceric ammonium nitrate concentrations: (□) 0.33×10^{-3} mole/l.; (○) 0.67×10^{-3} mole/l.; (△) 1.33×10^{-3} mole/l.

The results of qualitative carbohydrates determinations are summarized in Table III and show that there were neutral and amino sugars attached to the end of the grafted polyacrylamide. In order to check whether the grafted polyacrylamide was separated completely from the free glycopeptide, a blank test was made with the mixture of the homopolymer of acryl-

TABLE II
Grafting of Acrylamide onto Ovalbumin[a]

Sample	[Ceric salt], $\times 10^3$ mole/l.	Time, min.	Increase, %	Mol. wt. of grafted amide $\times 10^{-5}$	No. of grafting sites, mole/mole
Native ovalbumin	0.5	10	202	9.8	0.095
	1.0	5	136	8.0	0.077
	1.0	10	254	7.9	0.14
	1.0	15	265	7.9	0.15
	1.0	20	309	13.5	0.10
Heat-denatured ovalbumin in aqueous solution	2.0	4	458	7.1	0.30
	1.0	5	306	28.2	0.050
	1.0	10	640	31.0	0.095

[a] Reaction conditions: 30°C.; ovalbumin = 0.100 g.; acrylamide = 1.0 g.; [nitric acid] = 0.02 mole/l.; total volume = 20 ml.

amide and the authentic free glycopeptide. The free glycopeptide comes from the free ovalbumin which does not participate in grafting. Polyacrylamide used was prepared by a conventional method in aqueous redox system. The authentic free glycopeptide was prepared from ovalbumin by the method of Yamashina and Makino.[11] After dialyzing the mixture for 10 days, little carbohydrate was detected and dialysis was continued for another 20 days to make sure of this. Attempts to separate polyacrylamide from the free glycopeptide by gel filtration on Sephadex were unsuccessful.

TABLE III
Carbohydrates Attached to the Grafted Polyacrylamide

Sample	Anthrone test (neutral sugar)	Elson-Morgan test (amino sugar)
Blank[a]	±	−
Purified grafted polyacrylamide	+	+

[a] Mixtures of polyacrylamide and the free glycopeptide separated from ovalbumin.

DISCUSSION

In graft copolymerization onto ovalbumin by the redox process with ceric ion, the grafting initiation was extremely rapid and seemed to be complete within 5 min. under the conditions studied. This can be clearly seen from the results for the heat-denatured and dried ovalbumin, i.e., no significant change in per cent weight increase, molecular weight of grafted PMMA, and number of grafting sites was found within 5 to 15 min. Moreover, in the case of native ovalbumin, the number of grafting sites reached a maximum after about 2 min.

The remarkable phenomenon for the native ovalbumin can be attributed to the gel effect, in which a significant decrease in termination rate occurs as growing chains are occluded. A possible explanation for the results of Figure 1 are as follows. After the number of grafting sites has reached a maximum at about 400% weight increase, propagation of growing chains and termination by coupling occur at the same time, and then above a weight increase of about 700% only propagation of occluded growing chain radicals takes place. Grafting does not seem to proceed in a steady state as is the case with the usual radical polymerization. The apparent decrease in the number of grafting sites would be due to termination of growing chains by coupling and therefore, the actual number of grafting sites would agree with a maximum value. The graft copolymer would have one grafted side chain per ovalbumin molecule.

In ceric ion–alcohol redox systems, the molecular weight of the polymer formed is in inverse proportion to ceric ion concentration,[12] because ceric ion is responsible for both the initiation and the termination of the polymer

chain. But ceric ion in this grafting system does not seem to participate in the termination reaction, since a high ceric ion concentration caused rather slight increase in the molecular weight of grafted PMMA. A high ceric ion concentration may be unfavorable for grafting since the free radicals produced by the interaction between ceric ion and the protein are oxidized further before the initiation of grafting. The fact that the number of grafting sites has a maximum at a weight increase of about 400%, regardless of the initial ceric ion concentration, would suggest that the coagulation or the precipitation of the polymer begins to occur at that point.

The results of grafting may reveal that only a limited part of ovalbumin participates in the grafting. The preliminary experiments showed that proteins which are susceptible to grafting contain more or less carbohydrates, cysteine, and cystine. Ovalbumin consists of nineteen kinds of amino acids,[13] i.e., glutamic acid, serine, alanine, leucine, aspartic acid, valine, isoleucine, phenylalanine, lysine, glycine, threonine, methionine arginine, proline, tyrosine, histidine, cysteine, tryptophan, and cystine. Besides these amino acids, ovalbumin also contains a trace of oligosaccharide which consists of mannose and glucosamine and which is linked to the protein at the aspartyl residue.[11,14,15]

Very rapid grafting seemed to imply the participation of 1,2-glycol units in the grafting, as was suggested from the previous work.[3,4,16] As a working hypothesis, we assumed that the most likely grafting site on ovalbumin is the carbohydrate residue. If grafting results from participation of the carbohydrate residue it should be possible to block the carbohydrate prior to the polymerization. This was done by treating ovalbumin with periodic acid. Periodic acid is a specific oxidant for the various carbohydrates and has been reported to act in a manner similar to ceric salts.[17] The effect of periodic acid treatment appears clearly in the increase in molecular weight of grafted PMMA and shows a trend similar to that of ceric ion treatment (Table I). This means that periodic acid destroys the reducing sites of ovalbumin capable of forming the grafting sites and that the decrease in the reducing sites caused the increase of molecular weight of grafted PMMA. It has been reported that in periodate oxidation of ovalbumin the carbohydrate residue is oxidized relatively rapidly and that cysteine, cystine, methionine, tryptophan, and tyrosine residues are also eventually destroyed.[18] Therefore, the reducing sites affected by periodic acid treatment are involved in these six residues. But compared the compositions of amino acids of the proteins[13] used in the preliminary experiments, cystine, methionine, tryptophan, and tyrosine residues can be eliminated as common factors.

Blocking of the sulfhydryl group of cysteine with iodoacetic acid produced no significant change. The slight increase in the molecular weight of grafted PMMA and grafting sites may be due to the decrease of the sulfhydryl group which acts as a chain transfer agent in the radical polymerization.

These results demonstrate that the carbohydrate residue is a possible grafting site. This possibility is further enhanced by the fact that the neutral and amino sugars were detected at the end of grafted polymer molecule.

The detection of carbohydrate participating in the grafting site was successfully achieved for the first time by the aid of the proteolytic enzymes. Ovalbumin backbone was easily digested by the protease which has no effect on polyacrylamide and oligosaccharide. Whitfield et al.[19] have used papain to determine the grafting sites of the graft copolymer of wool–polyamide systems, but no satisfactory results have been obtained.

This study has revealed the important role of carbohydrates in rapid grafting by the ceric ion method.

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Résumé

La copolymérisation greffée du méthacrylate de méthyle et de l'acrylamide à l'ovalbumine a été effectuée au moyen d'un système rédox utilisant l'ion cérique. L'initiation du greffage se passe très rapidement et le nombre de sites de greffage atteignait un maximum après 2 min. indépendamment de la concentration initiale en ions cériques. Les chaînes polymériques greffées ont été séparées par hydrolyse sélective de la chaîne protéinique au moyen de l'acide chlorhydrique ou par la protéase âfin de caractériser les copolymères greffés. Le polyacrylamide greffé, séparé par digestion protéolitique, contenait des résidus d'hydrate de carbone à l'extrémité de la molécule polymérique. Le problème des sites de greffage à l'ovalbumine est soumis à discussion.

Zusammenfassung

Die Pfropfcopolymerisation von Methylmethacrylat und Acrylamid auf Ovalbumin wurde mit einem Redoxysystem unter Verwendung von Cer-IV-ionen durchgeführt. Der Start der Aufpfropfung erfolgte sehr rasch und die Zahl der Aufpfropfstellen erreicht, unabhängig von der Cer-IV-ionenkonzentration nach etwa 2 min ein Maximum. Zur Charakterisierung der Pfropfcopolymeren wurden die aufgepfropften Ketten durch selektive Hydrolyse der Eiweisskette mit Chlorwasserstoffsäure oder Protease abgetrennt. Das durch proteolytische Einwirkung abgetrennte Pfropfpolyacrylamid enthielt den Kohlehydratrest am Ende des Polymermoleküls. Das Problem der Aufpfropfstellen am Ovalbumin wird diskutiert.

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