Effects of Protein and Iron Concentrations on Iron Solubility in Black Tea Infusion*

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ABSTRACT

Tannins in plant foods and beverages may produce antinutritional or toxic effects although some proteins with high affinity for tannins seem to function as defense mechanism to tannin toxicity. Our objectives were to investigate possible interactions of tea tannins, iron and proteins and to evaluate the role of proteins in tannin effects on iron solubility. Iron solubility in vitro was measured using tea with and without proteins. Mixtures of tea, protein in varying concentrations(either gelatin or bovine serum albumin), and iron(either 10 or 50μg/mL) were prepared. Controls contained water in place of tea. Iron bioavailability was assessed by measuring iron solubility in the simulated gastric condition with pepsin digestion. Bound iron was removed by centrifugation and soluble iron was assayed using atomic absorption spectrophotometry. Iron was quite soluble in tea alone. When iron concentration was 10μg/mL, addition of small amounts of protein to tea dramatically reduced iron solubility, but solubility of iron increased in the tea mixtures as the concentration of protein was increased. The percentage of iron that precipitated was much greater at 10μg Fe/mL than the values at 50μg Fe/mL suggesting that the iron binding sites on the tea-protein complex was saturated. These results suggest that interactions of iron with tea tannins are influenced by the concentrations of protein and iron. (*Korean J Nutrition* 29(8) : 861~866, 1996)

KEY WORDS : iron · solubility · tea · tannin · protein.

Introduction

Tannins in foods have been associated with toxic and antinutritional effects including reduced food intake, growth retardation, and impaired nutrient absorption[1,2,3,4,5]. Drinking of tea with meals reduced nonheme iron absorption in several studies with rats[6,7] and humans[8,9]. Presumably, tannins in tea are responsible for this inhibition of iron absorption.

Proline-rich salivary proteins produced in response to dietary tannins protect against growth retardation and impaired protein utilization[10]. The presence of

proline rich proteins(PRP) in the saliva of several animals including humans has been known for some time[11]. The best evidence for their protective effect against tannins comes from feeding trials where PRP production was induced in rats[12] and mice[13] by feeding high tannin diets. Salivary PRP exhibit a very high relative affinity for tannins[12]. Hagerman and Butler[14] found that tannins selectively bound to PRP even in the presence of a large proportion of other proteins with marginal or average affinities for tannins.

We hypothesized that PRP may also protect animals against the iron-absorption-inhibiting properties of tannins by binding tannins and preventing them from complexing dietary iron. Since little is known about the effects of PRP on iron-tannin complex formation and stability, we measured the solubility of iron and

tannins in the presence of two selected proteins. Brewed black tea was used as the tannin source. Gelatin was chosen as a proxy for salivary PRP because of its high proline content, high affinity for tannins and ready availability. BSA was used as a control.

Materials and methods

1. Preparation of tea

One g of black tea(U.S. Tea Association, Black Tea Research Blend, Thomas J. Lipton Company, Englewood Cliffs, NJ) was added to 100 mL boiling distilled, deionized water(in an Elernmeyer flask) and left to stand at room temperature for 5min. The brewed tea was filtered(Whatmaı no.1 qualitative, Whatman Limited, England) and tannin concentration was determined by two methods: the 0.5% vanillin assay of Price et al.[15] and a method for determining the concentration of iron binding phenolic groups[16]. Tannic acid and catechin were used as standards.

2. Preparation of iron-tea-protein mixtures

Gelatin, type B from bovine skin(Approx. 75 Bloom, Sigma Chemical Co., St. Louis, MO, used as a proxy of PRP) and bovine serum albumin(BSA, Fraction V, Sigma Chemical Co.) were used as protein sources. Protein powder was added to 3 mL of tea in test tubes. The tubes were mixed on a vortex mixer and allowed to stand for 15 min(at 30°C for gelatin and at room temperature for BSA). Iron(Certified Atomic Absorption Standard, 1,000 ppm in 2% nitric acid, Fisher Scientific, Pittsburgh, PA) was added and the final volume for each tube was made to 5 mL with tea. Final iron concentrations were either 10µg/mL or 50µg/mL. Final protein concentrations were 0.2, 1, 2, 6, 12 or 20 mg/mL.

3. Solubility studies

Freshly prepared mixtures(see above) were allowed to stand 15 min at room temperature, then were centrifuged(5,000×g, 15min, Sorvall RC-5B, Dupont Instruments, Boston, MA) to remove insoluble complexes. Iron and tannin concentrations in supernatants were measured by atomic absorption spectrophotometry (Perkin-Elmer, Norwalk, CT) and the vanillin assay, respectively.

In an attempt to simulate conditions present in the stomach following tea ingestion, the tea-protein-iron mixtures were subjected to pepsin digestion. Water or tea with added protein and iron were prepared and

held for 10min. One half mL of a pepsin suspension (pepsin A from porcine stomach mucosa, Sigma Chemical Co., 8mg/mL suspended in 0.01 N HCl) was added to each tube and the final volume was made to 5mL with water or tea yielding 10µg Fe/mL or 50µg Fe/mL, and protein concentrations varying from 0 to 20mg/mL. The tubes were vortex mixed and incubated at 37°C in a shaking water bath for 90min. After incubation, insoluble complexes were removed by centrifugation using a bench top IEC clinical centrifuge(International Equipment Company, Nedham Heights, MA) run at maximum speed(2575× g) for 15 min. Concentrations of iron and tannin in supernatants were measured as described earlier.

Proteins may also bind iron and may compete with tannins for iron. Therefore, solubilities of iron in protein solutions were estimated. Protein solutions were prepared by dissolving 0 to 100 mg of proteins in 5 mL of deionized distilled water. Buffer was not used in order to avoid interactions between buffer salts and iron. Iron was added to make final concentrations of 10µg Fe/mL or 50µg Fe/mL. The pH of each solution was measured after addition of iron. Insoluble complexes were removed by centrifugation(2575×g, IEC clinical centrifuge, International Equipment Company, Needham Heights, MA). Concentrations of iron in supernatants were measured as described earlier. Pepsin digestion was also conducted for protein-iron solutions.

Results

Tannin concentrations in the tea are shown in Table 1. The vanillin assay is specific for the resorcinol group in flavanols and flavanoids. Catechin is used as the standard and the assay does not detect the galloyl groups. Discrepancies in values for catechin equivalents are due to different specificities of the two methods[16].

Effects of protein and iron concentrations on iron solubility in tea and water are shown in Figures 1 and 2. In the absence of protein, iron was highly soluble

Table 1. Concentrations of tannins in tea[1]

Method used	Catechin equinvalents	Tannic acid equinvalents
	µg / mL	
Vanillin assay[2]	140.0±7.1	—
Iron binding assay[3]	118.5±1.1	82.0±2.5

[1]Values are means±SD, n=3
[2]Calaulated as price et al(1978).
[3]Calculated as Brune et al(1991).

in both tea and water at both iron concentrations. Addition of protein to water reduced iron solubility somewhat in all solutions. In the 10μg/mL iron solutions, iron solubility in tea was dramatically affected by adding small amounts of protein, but solubility increased with increasing protein concentrations(Fig. 1). In the case of gelatin(Fig. 1A), this increase in iron solubility was abolished by pepsin digestion. In contrast, pepsin digestion had little effect in the BSA treatments(Fig. 1B).

At higher iron concentrations(50μg Fe/mL), added protein and pepsin digestion had less of an effect on iron solubility than was the case at lower iron concentrations, regardless of the protein used. Moreover, digestion had little or no effect(Fig. 2).

Fig. 3 shows that iron solubility follows tannin solubility. As with iron, tannin solubility increased with increasing concentrations of added gelatin. Pepsin digestion caused precipitation of both tannin and iron at all protein concentrations.
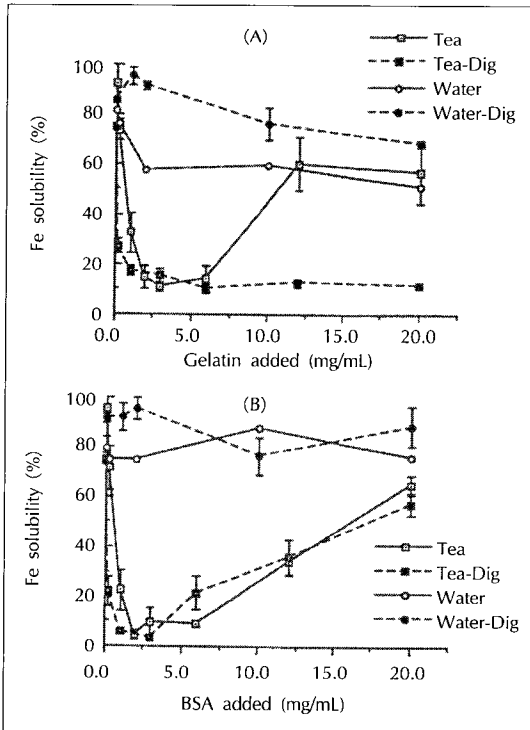
Iron precipitation(expressed as total Fe in precipitate) increased with iron concentration in tea/gelatin mixture but reached a maximum at about 30μg Fe/mL(Fig. 4). This effect may be different with different proteins and protein concentrations.
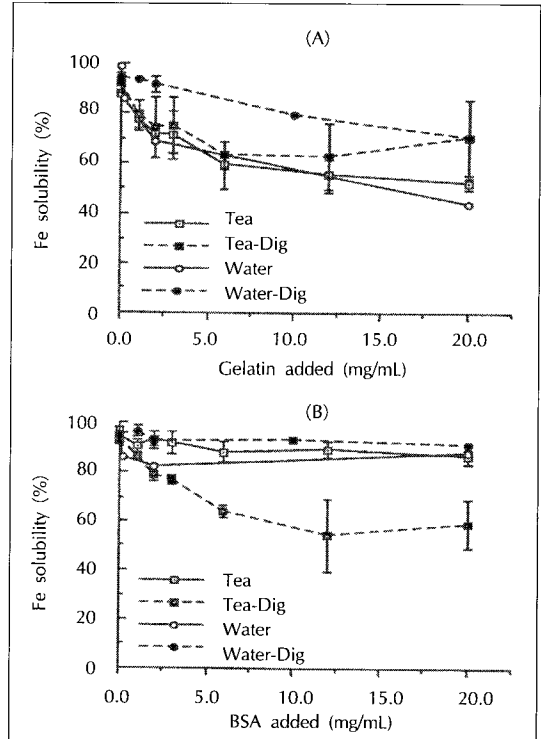
Fig. 2. Relationships between concentration of protein (gelatin or BSA) and iron solubility in tea/protein and water/protein solutions(50μg Fe/mL). Tea : final pH=2 − 3 ; Tea-Dig : final pH=2 − 3 ; Water-Dig : final pH=2 − 3. Values are means with SD as error bars(n=3).

Fig. 1. Relationships between concentration of protein (gelatin or BSA) and iron solubility in tea/protein and water/protein solutions(10μg Fe/mL). Tea : tea+ protein+Fe, no digestion, final pH=2 − 4 ; Tea-dig : same as Tea but 90min pepsin digestion, final pH= 2 − 3 ; Water : same as Tea except water replaced tea, final pH=3 − 4 ; Water-dig : same as Tea/dig except water replaced tea, final pH=2 − 4. Values are means with SD as error bars(n=3).
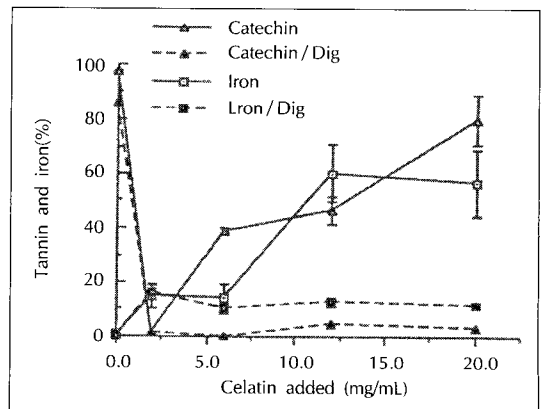
Fig. 3. Tannin solubility in tea/Fe mixtures containing varying amounts of added gelatin. Iron concentration was 10μg Fe/mL ; pH=3.0 − 3.5. Values are means with SD as error bars(n=3).
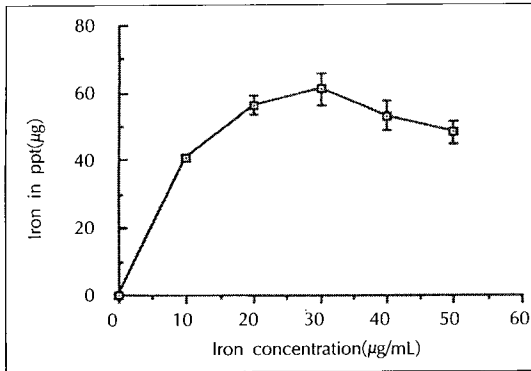
Fig. 4. Amount of iron in precipitates of tea / gelatin mixtures containing varying amounts of added iron. Total volume=5mL ; gelatin concentration=6mg/mL ; pH=3.0 – 3.5. Values are means with SD as error bars(n=3).

Discussion

This research has attempted to assess the effect of tea tannins on the solubility of iron. Tannins were not separated from other components of tea since extraction processes may change the chemical structures of tannins and other polyphenols. Transformed tannins may behave differently from those present in brewed tea. Tea contains phenolic compounds other than tannins and it is possible that some phenolic compounds other than tannins may interact with iron as well. However, only tannins possess the ability to precipitate protein[17]. Moreover, it was shown previously that tannin was the main cause of the inhibitory effect of tea on iron absorption[18]. The objective of this study was to investigate interactions among protein, tannins and iron. Therefore, we have chosen to use the term 'tannin' although we could not clearly eliminate the possibility that other components of the tea were involved in the interactions with iron in these experimental conditions.

The results may be summarized by pointing out that substantial iron precipitation occurred only when protein was present in the tea. Moreover, when expressed on a percentage basis, iron precipitation was greater when iron concentrations were low. This may be attributed to the ability of tannins to form an insoluble tannin-protein complex capable of binding a limited amount of iron.

Presumably, Haslam and Lilley[17] and Haslam[19] proposed a model to explain the behavior of protein-tannin mixtures. When tannins interact with proteins, they form a relatively hydrophobic layer on the surface of
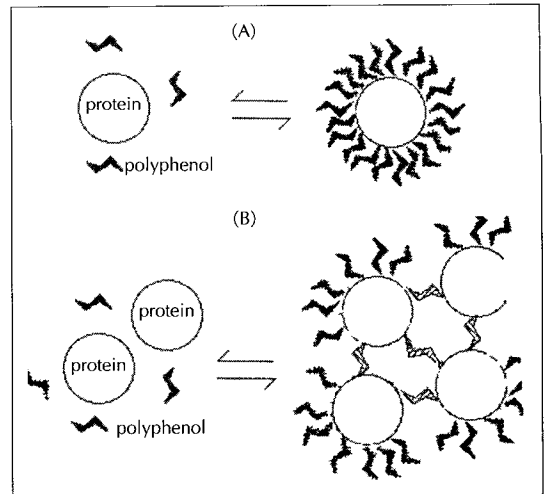

Fig. 5. Pictorial representation of protein-polyphenol precipitation(Redrawn from Haslam and Lilley 1985). (A)At low protein concentration. (B)At high protein concentraiton.

the proteins. This leads to aggregation and precipitation(Fig. 5). At low protein concentrations, the protein surface is covered with tannins to give a monolayer that is less hydrophilic than the protein itself(Fig. 5A) giving rise to aggregation and precipitation. When protein concentrations are high, cross-linking of different protein molecules by the multi-dentate tannins occurs in addition to the formation of a hydrophobic surface layer(Fig. 5B). Precipitation then follows as above. Therefore, more tannin is required to precipitate proteins from dilute protein solutions than from concentrated solutions.

This model is helpful in explaining our observations that the addition of small amounts of protein to iron-tea mixtures caused most of the iron to precipitate while higher protein concentrations resulted in increasing iron solubilities(Fig. 1). Pepsin digestion prevented this increase in the case of gelatin but not BSA. Presumably, iron binds to protein-tannin complexes and is carried into the precipitate. As more protein was added, less tannin was used for tannin-protein complex formation leaving free tannins as soluble ligands for iron. Therefore, both gelatin and BSA showed more soluble iron as more protein was added at the 10μg Fe/mL.

With pepsin digestion, proteins were hydrolyzed to smaller peptides giving a larger total surface area. Therefore, these smaller digestion products of protein required more tannins to form complexes even when protein concentrations were higher. Thus, all the tan-

nins precipitated leaving no soluble tannins to main-
tain iron in solution. BSA has a larger molecular
weight(60,000) than the gelatin used in these ex-
periments(20,000 - 25,000). Thus, BSA digestion pro-
ducts are likely to be larger than digestion products of
gelatin, resulting in a smaller surface area than gelatin.
Therefore, less tannin might be needed with BSA
digestion products for forming insoluble tannin-pro-
tein complexes than with gelatin digestion products.
This may explain why digestion of BSA did not affect
iron solubility the way digestion of gelatin did.

At a high iron concentration(50μg Fe/mL, Fig. 2),
the extremely low iron solubility at low protein con-
centrations with 10μg Fe/mL was not observed. This
implies the formation of soluble iron-tannin com-
plexes regardless of the amount of added protein.
These complexes could be smaller than the complexes
formed in the case of low iron concentration(10μg Fe/
mL) and soluble because iron bound to the mono-
layer tannin surface of protein-tannin complex pre-
vented hydrophobic aggregation.

There appears to be a finite iron binding capacity
for tannin-protein complexes beyond which iron-tan-
nin-protein binding does not occur. The binding sites
of tannin at the surface of tannin-protein complexes
seemed to be saturated by iron and precipitated at
30μg Fe/mL with 6mg/mL gelatin. When more iron
(40 or 50μg Fe/mL) was used, excessive iron may
have bound to tannins before tannins bound to pro-
tein, which possibly reduced aggregation or cross-link-
ing of protein molecules by tannins. As a result, re-
latively small soluble complexes may have been pro-
duced. This suggests that iron-tannin-protein complex
formation is highly concentration dependent. De-
pending on the ratio of iron : tannin : protein, either
soluble or insoluble complexes can be formed[18]. The
solubility of iron-tannin-protein complexes seems to
be determined by several physico-chemical states of
each component such as concentration of each com-
ponent, molecular weight of protein, binding affinity
and size of complexes formed.

This study has taken a step in the direction of de-
fining the relationship between iron, tannin and pro-
teins, especially PRP and its consequences to iron ab-
sorption. It is possible of course that normal gas-
trointestinal tract conditions may be entirely different
from in vitro conditions used in this study since tea is
mostly consumed with or after meals and varieties of
dietary components will change the gastrointestinal

tract condition.

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=국 문 초 록=

단백질 함량 및 철분 농도의 변화에 따라 홍차 추출물이 철분의 용해도에 미치는 영향

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식물성 식품이나 음료에 함유되어 있는 탄닌성분은 영양학적으로 여러 가지 문제를 야기하는 것으로 알려져있다. 그러나 탄닌에 대한 결합력이 높은 단백질은 이러한 탄닌성분이 초래하는 문제점들을 상쇄시킬 수 있으며, 인간을 포함한 몇 종의 동물들은 탄닌에 대한 결합력이 높은 단백질을 타액으로 분비함으로써 탄닌에 대한 방어능력을 가진 것으로 보여진다. 본 연구는 홍차내의 탄닌성분과 단백질의 반응성이 철분의 용해도에 미치는 영향에 대하여 조사하였다. In vitro에서의 철분의 용해도(solubility)를 홍차 추출물과 여러 농도의 단백질(gelatin 또는 bovine serum albumin) 및 철분(10μg/mL or 50μg/mL)이 함유된 혼합물에서 측정하였다. 철분의 생체이용률은 위장에서의 상태와 비슷한 환경을 조성하기 위하여 pepsin digestion 과정을 거쳐 용해도를 측정함으로써 조사하였다. 탄닌-단백질과 결합된 철분은 원심분리에 의해서 제거하고 용해되어 있는 상태의 철분만 atomic absorption spectrophotometry로 측정하였다. 홍차추출물에 단백질이 첨가되지 않은 상태에서는 철분의 용해도가 상당히 높았으나, 10μg/mL의 철분농도에서 단백질이 소량 첨가되면 많은 양의 철분이 침전되었다. 그러나 첨가되는 단백질의 양이 늘어날수록 철분의 용해성은 점차 커졌다. 소량의 단백질에 의해서 침전되는 철분의 비율은 10μg Fe/mL 용액에서가, 50μg Fe/mL 용액에서 보다 컸다. 이러한 현상은 홍차내의 탄닌과 첨가된 단백질의 결합체에 철분이 결합되는 정도가 제한되어 있기 때문인 것으로 볼 수 있겠다. 따라서 본 연구의 결과, 홍차내 탄닌 성분이 철분의 용해도에 미치는 영향은 함께 존재하는 단백질의 양이나 철분의 양에 따라 달라지는 것을 볼 수 있다.